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OCCURRENCE OF PSEUDOMONAS AERUGINOSA (SCHROETER) MIGULA IN HAEMOLYMPH OF GRASSHOPPERS AFTER INFECTION BY FEEDING[1]

JUNE M. STEPHENS[2]

Abstract

When adults of the grasshopper *Melanoplus bivittatus* (Say) were fed large doses of *Pseudomonas aeruginosa* (Schroeter) Migula, the haemolymph remained sterile for various lengths of time. Most of the infected grasshoppers first carried small numbers of *P. aeruginosa* in the haemolymph and all but one died in 1 to 3 days after its first appearance in the haemolymph.

Introduction

The course of infection in grasshoppers fed large doses of *Pseudomonas aeruginosa* (Schroeter) Migula was studied in an attempt to determine when and the degree to which the blood of these insects becomes infected before death. Though most descriptions of bacterial diseases of insects refer to heavy infection of the haemolymph, or blood, before death, few of these give the number of bacteria present or the duration of the septicemia. Steinhaus (4) pointed out that septicemia may precede death by only a few hours or may persist for several days before the death of the insect.

Determination of the per os LD_{50} of *P. aeruginosa* for adult grasshoppers showed that a long interval often occurs between ingestion of the bacteria and death from the disease (1). During this interval the bacteria evidently exist in small numbers in the gut and eventually gain access to the haemocoel.

Materials and Methods

Three groups of adult grasshoppers, *Melanoplus bivittatus* (Say), from different laboratory colonies were starved for 1 day, and then individually fed lettuce contaminated with approximately 10^6 *P. aeruginosa* organisms. This number is 35 to 120 times the calculated LD_{50} (1) and is sufficiently large to ensure infection of a high percentage of the experimental insects. All experimental grasshoppers were maintained individually in cardboard containers

[1]Manuscript received January 16, 1958.
Contribution No. 3738, Entomology Division, Science Service, Department of Agriculture, Ottawa, Canada.
[2]Entomology Laboratory, Belleville, Ontario.

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as preliminary experiments showed that the percentage mortality and speed of infection were greatly increased if grasshoppers were maintained in small vials.

Samples of haemolymph were taken from each insect before feeding, to check on the sterility of normal haemolymph, and after feeding, to detect the first appearance of *P. aeruginosa*, by inserting a sterile capillary tube in the pleural membrane in the area of the fourth to seventh abdominal segments. Samples of blood ranging from 0.01 to 0.05 ml. were obtained, depending on the size of the grasshoppers. The capillary tube was crushed in 5.0 ml. of Bacto-nutrient broth (Difco).

To determine the minimum limit of the number of bacteria in the blood of a grasshopper, one 0.01-ml. loop of the mixture was cultured immediately and a second after 24 hours' incubation. If a 0.01-ml. sample showed any growth when cultured immediately, the grasshopper carried at least 50,000 bacteria per milliliter of blood; if the sample did not show growth until after incubation, the insect carried at least 100 bacteria per milliliter of blood. The ratio of blood volume of grasshoppers to body weight is unknown but it may be assumed, from determinations made on other insects, to be between 25 and 40 to 100 (3). The specific gravity of *M. bivittatus* haemolymph is 1.027 (2) and the mean weight of the experimental grasshoppers was 1 g. The blood volume then probably ranged from 0.26 to 0.42 ml. and the minimum number of bacteria carried by a grasshopper was considerably less than 100 or 50,000.

The haemolymph was examined daily for 1 month. After death, all grasshoppers were triturated and cultured for the presence of *P. aeruginosa*.

Results

Table I shows that, of the 87 grasshoppers infected, 24% had no detectable bacteria in the haemolymph 24 hours before death; 43% had first evidence of bacteria on the day before death; 21%, 2 days before; 9%, 3 days before; and 3%, 4 days before. Fifty-four of the infected grasshoppers died within 7 days after feeding, 24 in 8 to 14 days, and nine in 15 to 21 days.

TABLE I

TIMES OF INFECTION AND MINIMUM NUMBERS OF BACTERIA IN HAEMOLYMPH OF ADULTS OF THE GRASSHOPPER *Melanoplus bivittatus* AFTER BEING FED LARGE DOSES OF *P. aeruginosa*

No. of grasshoppers	No. lethally* infected with *P. aeruginosa*	No. with sterile blood 24 hr. before death	No. with *P. aeruginosa* in blood: Minimum no. of bacteria per ml. of haemolymph: 100 — Days before death: 4	3	2	1	50,000 — Days before death: 4	3	2	1
55	42	11	1	2	4	17	0	2	0	5
34	18	6	0	0	1	6	0	1	1	3
40	27	4	2	3	6	4	0	0	6	2
Total 129	87	21	3	5	11	27	0	3	7	10

*One individual showed *P. aeruginosa* in blood but recovered.

The blood of all grasshoppers was sterile before they were fed *P. aeruginosa*. In several instances, nonproteolytic bacteria were found in the haemolymph after feeding but grasshoppers always recovered from such infections. It is assumed that these invading species are normal inhabitants of the gut. With one exception shown in Table I, grasshoppers did not recover after *P. aeruginosa* organisms were noted in the blood. In those insects that showed a minimum of 100 bacteria per milliliter of blood 2 to 4 days before death, a greater number of *P. aeruginosa* were generally present in the haemolymph on the day after original detection and a positive plate was obtained without incubation of the sample. Similarly, a rapid increase in number of bacteria must have occurred in a few grasshoppers that showed a minimum of 100 bacteria per milliliter of haemolymph when the sample was taken and yet were dead about six to eight hours later.

Previous work had shown that *P. aeruginosa* cannot be isolated from the gut of grasshoppers by routine-plating procedures 72 hours after ingestion (1). In a further attempt to determine whether the bacteria are detectable anywhere in the insect 72 hours after ingestion, 30 grasshoppers were fed approximately 10^6 bacteria and 3 days later each insect was completely crushed and plated. Again no *P. aeruginosa* organisms were detected.

Discussion

In the grasshoppers that survived after being fed a large dose of *P. aeruginosa* (33%), the bacteria were either destroyed in the gut or overcome by the natural defences of the insect. As the bacteria were not detectable anywhere in the grasshopper after 72 hours, it was impossible to predict where they remained until they invaded the haemocoel. It is apparent that *P. aeruginosa* is not actively invasive, but under proper conditions can produce infection in the grasshopper. The speed of infection and percentage mortality were greatly increased if grasshoppers were maintained in vials where space was limited and humidity was high.

In 67% of the grasshoppers killed, *P. aeruginosa* was first detected in the blood only 24 hours before death or not at all. There are no apparent external signs of infection until death and the insects generally die before the presence of the organism in the blood is proved by existing techniques. Therefore it is impossible to detect the time or to study the course of invasion more closely without increasing the number of blood samples to such an extent that the sampling alone might be injurious to the insect.

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THE PRODUCTION OF POLYSACCHARIDES BY FUNGI ACTIVE IN THE DECOMPOSITION OF WOOD AND FOREST LITTER[1]

B. BERNIER[2]

Abstract

Four fungi known to be active in the decomposition of wood and forest litter were grown on a liquid medium containing salts and glucose and also on aqueous extracts of a variety of fresh leaves and litter. The polysaccharides produced in the various culture media by the same organism were found, by chromatography of the hydrolyzates, to be of the same composition. In some cases, when the fungi were grown on leaf or litter extracts, polysaccharide synthesis did not take place because of lack of carbohydrate material. In many instances polysaccharide synthesis was inhibited by a factor or factors present even in much diluted extracts.

Introduction

Decomposition of vegetable debris by microorganisms is often accompanied by the synthesis of extracellular compounds. Some of these products of microbial synthesis, particularly polysaccharides, have been credited with having a beneficial effect on the physical properties of soil (6, 18). Considerable indirect evidence has been accumulated in support of this. However, little has been done to determine whether microorganisms active in the breakdown of forest litter might vary in their ability to synthesize polysaccharides when developing on various types of vegetable debris. The present work was intended as a step towards the elucidation of this point and was suggested by the observation made by Handley (8) that certain fungi (four of which have been used in the present investigation) were capable of synthesizing considerable amounts of water-stable jellies when grown on aqueous extracts of plant debris.

This paper, therefore, reports the results of an exploratory investigation of polysaccharide production per se by four fungi when grown on aqueous extracts of fresh leaves or litter of a variety of species which are known to be associated with the formation of either mull or mor types of humus. Some observations on the composition of these polysaccharide materials are also reported.

Material and Methods

Organisms

The four fungi used in the present investigation are the following: *Pullularia pullulans*, *Polystictus versicolor*, *Lenzites betulina*, and *Marasmius dryophilus*. They were obtained from the culture collection of the Imperial Forestry Institute, Oxford.

[1]Manuscript received August 15, 1957.
Contribution from the Department of Forestry, University of Oxford, Oxford, England.
[2]Present address: Faculty of Forest Engineering, Laval University, Quebec, Que.

Media and Cultural Conditions

Essentially two types of media were used: (*a*) an artificial medium containing salts and glucose, and (*b*) several media formed by aqueous extracts of plant debris. The former medium was used to obtain more easily purified polysaccharide materials for analytical purposes, and to which the polysaccharides produced by the same fungus, when grown on the latter media, could be compared.

(*a*) *Medium Containing Salts and Glucose*

This consisted of the Czapek–Dox medium containing 5% (w/v) glucose. To each cultural flask containing 200 ml. of medium, 5 ml. of a 1% (w/v) "Yeastrel" solution (yeast extract from Brewers' Food Supply Co. Ltd., Edinburgh) was added. The salts, glucose, and yeast extract were sterilized separately, the latter by filtration. The fungi were grown as stationary cultures in the dark at 21° C. for 20 days on shallow layers of medium (in conical flasks of 2 l. capacity).

(*b*) *Culture Media Consisting of Aqueous Extracts of Fresh Leaves or Litter*

The air-dried leaves (collected from the tree late in summer) or litter (freshly fallen leaves) of a variety of forest trees and *Calluna vulgaris* were used for extraction. To 100 g. of milled leaves or litter, 400 ml. of distilled water and 5 ml. of chloroform were added, and extraction was carried out at room temperature in sealed jars for 1 week, with occasional shaking. The extracts were separated from the solid material by means of a press and then filtered. The clear solutions were sterilized by autoclaving, which also effected removal of most of the chloroform. Substrates for the growth of microorganisms were subsequently prepared from these extracts. The fungi were grown as stationary cultures for 20 days in the dark at 21° C. in 50-ml. Erlenmeyer flasks containing 15 ml. of the fresh leaf or litter extracts. Polysaccharide production per se could be easily detected by the immediate formation of a filamentous precipitate upon addition of 1.5 volume of ethanol to a sample (3 ml.) of the culture fluid removed aseptically. This concentration of ethanol was not sufficient to precipitate any of the plant polysaccharides contained in the extracts.

Isolation of the Polysaccharides for Analysis

The fungal hyphae and adherent jelly were removed by filtration and carefully washed. The resulting filtrate was dialyzed at 4° C. for 2 days, against many changes of distilled water, and then concentrated under reduced pressure at 20°–25° C. Proteins were removed by shaking with Sevag's mixture (17). The very small amount of gel formed during this treatment was removed by centrifugation. The polysaccharides were then easily isolated by addition of 1.5 volume of ethanol followed by gentle stirring with a glass rod to which the filamentous precipitate adhered. Alternate solution in water and precipitation with cold ethanol was repeated several times. The precipitate was next washed with ethanol and then repeatedly with ether, and allowed to dry in the air. This procedure, at least when used with polysaccharides produced in the Czapek–Dox medium, resulted in some purification.

This method has been used for the isolation and purification of the extracellular polysaccharides of some *Aerobacter–Klebsiella* strains (3, 19).

The failure of attempts to dissolve the jellies adherent to the mat of mycelium by mechanical shaking in cold water or sodium chloride solutions indicated that more drastic procedures were necessary in order to effect solution. The jellies were suspended in several volumes of water, the reaction being adjusted to pH 7.0 when necessary, and the suspension was heated on a boiling-water bath for 15 minutes. The jellies could be subsequently dissolved by mechanical shaking in water. A similar method has been used for the extraction of the capsular material from certain bacteria (3, 7, 19).

Methods Used in the Examination and Analysis of the Polysaccharides

Total nitrogen and phosphorus.—These determinations were carried out by Weiler and Strauss (Microanalytical Laboratory, Oxford).

Hydrolysis.—The polysaccharides (10–20 mg.) were best hydrolyzed in water in the presence of 12 times their weight of a cationic exchange resin (Amberlite IR-120) in sealed glass tubes at 97°–100° C. for 18 hours (1, 4). After separation of the hydrolyzates from the resin and washing of the resin, the combined solutions were evaporated *in vacuo* to dryness and the residue further hydrolyzed with N H_2SO_4 for 6 hours at 97°–100° C. Neutralization was effected with barium carbonate.

Paper chromatography.—This was carried out at room temperature on Whatman filter papers No. 1 and No. 3MM, using butanol–pyridine–water (10:3:3, by volume) (12), or ethyl acetate – acetic acid – water (3:1:3, by volume) (10) as solvent systems. The position of the sugars on the chromatograms were revealed by spraying with *p*-anisidine hydrochloride (9) or with aniline phthalate (14). The term R_g used in the text refers to the rate of movement of the spots relative to glucose.

Free-boundary electrophoresis.—Experiments were carried out at 4° C. by standard techniques, using the standard Hilger equipment, with the optical system of Philpot (15).

Results

(*a*) *Observations on the Chemical Composition of the Polysaccharides*

The polysaccharides produced by a given organism growing on the various media formed by aqueous extracts of plant debris were found, on analysis of the hydrolyzates by paper chromatography, to be of the same composition as those produced by the same organism growing on the Czapek–Dox medium. For this reason, and also because the polysaccharides isolated from this artificial medium were more easily purified, only the latter preparations were subjected to the analysis whose results are reported hereunder.

All the polysaccharides were isolated as gray-white stringy masses which dissolved in water with great difficulty to give highly viscous solutions. Unless special precautions were taken to avoid excessive drying during the course of the isolation procedures they became markedly insoluble in water

(at 100° C.), and even in alkali. For this reason, their specific rotations and acid equivalents could not be determined. The concentrations stated as being used in the electrophoresis investigations are only approximate. The polysaccharides gave no coloration with iodine and did not reduce Fehling's solution. No characteristic ketose coloration was produced on treating with the anthrone reagent of Johanson (11). They all gave a positive carbazole test (2) for uronic acids. The biuret test was negative. Nitrogen and phosphorus contents are shown in Table I.

TABLE I

NITROGEN AND PHOSPHORUS CONTENTS OF SOME OF THE FUNGAL POLYSACCHARIDES

Organisms	N, %	P, %
Pullularia pullulans	0.96	3.74
Pullularia pullulans (jelly)	0.67	0.41
Lenzites betulina	0.43	1.50
Polystictus versicolor	0.32	3.03

Jellies adherent to the mat of mycelium were produced by all the organisms used; but it was only in the case of *P. pullulans* that the polysaccharide material of the jelly was found, on analysis of the hydrolyzates by chromatography, to be different in composition from the polysaccharide preparation obtained from the culture fluid. It is therefore possible that the polysaccharide material of the jellies dissolves slowly into the medium during the growth of the organisms.

When examined in the free-boundary electrophoresis apparatus in borate buffer (pH 9.05, I 0.12) the polysaccharide preparations obtained from the culture medium were found, with the exception of *P. pullulans* which gave two distinct peaks, to give a single displacing boundary (Fig. 1). This is not, however, a definite criterion of homogeneity since it is known that polysaccharides are not always easily separated by electrophoresis.

Attempts to free the polysaccharide material of *P. pullulans* from the contaminating jelly-like polysaccharide by the use of ethanol fractionation procedures were not completely successful. Some of the glucose and uronic acid mentioned in Table II among the sugar components of the polysaccharide material may, therefore, be contaminating material originating from the jelly. The jelly, however, could be obtained free from other polysaccharide contaminants. The suspension of this jelly in water required heating for at least 30 minutes on a boiling-water bath to effect subsequent dissolution.

Chromatography of the hydrolyzates of the various polysaccharide preparations suggested that they may well be formed by a mixture of components, because of the multiplicity of the constituent sugars present. For this reason, quantitative estimation of the component sugars was not attempted but a subjective estimate of the relative amounts of the various sugars present in the hydrolyzates is shown in Table II. Paper chromatography alone does not

PLATE I

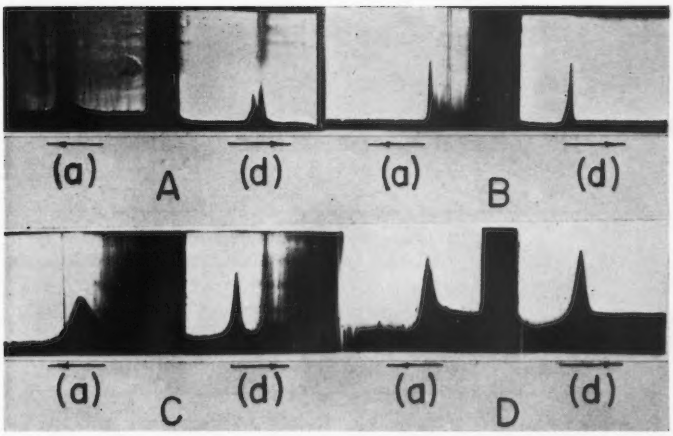

FIG. 1. Electrophoretic patterns of some fungal polysaccharides in borate buffer (pH 9.05, *I* 0.12); potential gradient, 5.2 v. cm.$^{-1}$. Direction of movement anodic as shown by arrows; (*a*), ascending boundary; (*d*), descending boundary.
(A) *Pullularia pullulans*: 0.25% polysaccharide, exposure at 68 minutes.
(B) *Polystictus versicolor*: 0.3% polysaccharide, exposure at 61 minutes.
(C) *Lenzites betulina*: 0.4% polysaccharide, exposure at 106 minutes.
(D) *Marasmius dryophilus*: 0.4% polysaccharide, exposure at 87 minutes.

TABLE II

THE CONSTITUENT SUGARS OF SOME FUNGAL POLYSACCHARIDES

Organisms	Hexoses			Pentoses	Methyl pentoses	Uronic acids
*Pullularia pullulans**	Galactose (t)	Glucose (M)	Mannose (m)	—	Rhamnose (t)	Glucuronic
Pullularia pullulans (jelly)	—	Glucose (M)	—	—	—	Glucuronic
Marasmius dryophilus	Galactose (t)	Glucose (M)	Mannose (m)	Xylose (m)	—	Glucuronic
Lenzites betulina	Galactose (t)	Glucose (M)	Mannose (M)	Xylose (m)	Fucose (m) Rhamnose (t)	Glucuronic
Polystictus versicolor	Galactose (t)	Glucose (M)	Mannose (m)	Xylose (m)	Fucose (t) Rhamnose (t)	Glucuronic

NOTE: (M), major component; (m), minor component; (t), trace.
*This preparation was slightly contaminated by the jelly.

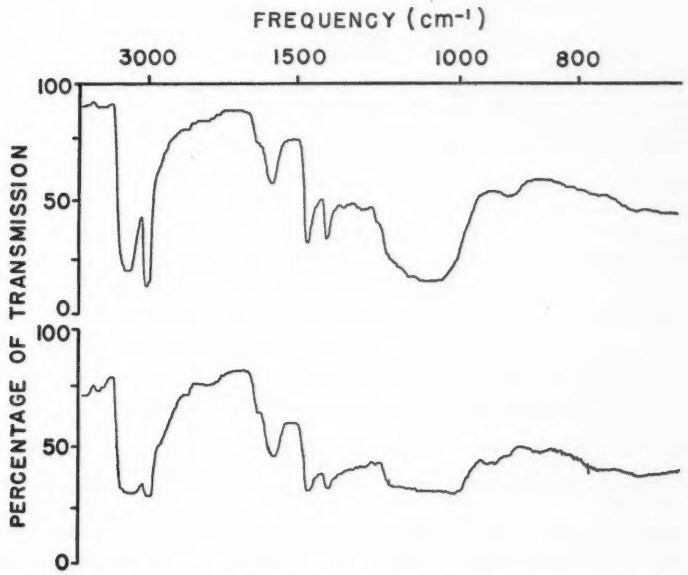

FIG. 2. Infrared spectra of the polysaccharides produced from glucose by *Polystictus versicolor* (top) and *Pullularia pullulans* (bottom).

in itself constitute a positive identification but at least suggests which sugars were present in the hydrolyzates. The uronic acid has been identified as glucuronic acid because of the presence of its lactone on the chromatograms when developed with an acidic solvent. Infrared spectra obtained for the polysaccharides produced from glucose by *P. pullulans* and *P. versicolor* (Fig. 2) confirmed the presence of carboxyl groups arising from the small amount of glucuronic acid residues (13).

(*b*) *Polysaccharide Production per se by Fungi Growing on Aqueous Extracts of Fresh Leaves or Litter*

Subjective estimates of the polysaccharide production per se by fungi growing on undiluted extracts of fresh leaves or litter are shown in Table III. Under the standard conditions of test (incubation for 20 days), *P. pullulans* produced polysaccharide on two of five extracts, *M. dryophilus* on one, and *L. betulina* and *P. versicolor* on none.

TABLE III

POLYSACCHARIDE PRODUCTION BY FUNGI GROWN ON UNDILUTED EXTRACTS OF FRESH LEAVES OR LITTER

Organisms	Litter extracts‖				
	Calluna* (*Calluna vulgaris* (L.) Hull.) (0.67)‡ (3.4)§	Beech*,† (*Fagus sylvatica* L.) (0.58)‡ (4.7)§	W. elm** (*Ulmus glabra* Huds.) (2.28)‡ (5.8)§	Ash** (*Fraxinus excelsior* L.) (1.98)‡ (4.9)§	Hazel** (*Corylus avellana* L.) (0.44)‡ (5.2)§
Pullularia pullulans	+++	+++	−	−	−
Lenzites betulina	−	−	−	−	−
Polystictus versicolor	−	−	−	−	−
Marasmius dryophilus	+++	−	−	−¶	−¶

*Mor-forming species;**mull-forming species.
†Fresh leaf extract.
‡Total nitrogen content of the extract, in mg./10 ml. of crude extract.
§pH of extract.
‖−, no polysaccharide production; +, little production; ++, moderate production; +++, abundant production (subjective estimates, after 20 days incubation).
¶Moderate polysaccharide production (++) after incubation for 2 months.

An important factor to be considered in the present case is whether there was sufficient carbohydrate material present initially in the extracts to permit polysaccharide synthesis. No attempt was made to estimate the amount of reducing sugars present but all solutions gave a strongly positive anthrone

reaction and reduced Fehling's solution. Another aspect deserving consideration is that polysaccharide synthesis may be, to a certain extent, prevented by too great a concentration of nitrogenous compounds (5, 20). In order to obtain information as to whether such factors, or others, were influencing polysaccharide production, the fungi were grown on a series of dilutions of the original extracts, in the presence and absence of added glucose. Details of cultural conditions and subjective estimates of polysaccharide production per se are presented in Tables IV to VII.

TABLE IV

POLYSACCHARIDE PRODUCTION BY *P. pullulans* WHEN GROWN ON AQUEOUS EXTRACTS OF FRESH LEAVES OR LITTER

Dilutions§	Litter extracts				
	Calluna*	Beech	W. elm	Ash	Hazel
1:0	+++	+++	−‡	−	−
1:0 (G)	+++	+++	++‡	−	+++
1:1	+++	++	−	−	−
1:1 (G)	+++	+++	++	−	+++
1:3	+++	+	−	−	−
1:3 (G)	+++	+++	++	−	+++
1(1:3):1 Call. (1:3)		+++	+++	+	+++
1(1:3):1 Call. (1:3) (G)		+++	+++	+	+++
1:7	++	−	−	−	−
1:7 (G)	+++	+++	+++	+++	+++
1(1:3):10 C.D. (G)	+++†	+++†	+++†	+++†	+++†

*Fresh leaf extract.
†Abundant jelly production.
‡Little fungal growth.
§1:0, undiluted litter extract (100 g. litter extracted with 400 ml. water); 1:1, etc., one volume of extract diluted with one volume of distilled water, etc.; (G), glucose added aseptically to give a final concentration of 5%; 1(1:3):1 Call. (1:3), one volume of extract (dil. 1:3) with one volume of calluna extract (dil. 1:3); 1(1:3):10 C.D., 1 ml. of extract (dil. 1:3) with 10 ml. Czapek–Dox medium.

NOTE: Subjective estimates after 20 days incubation.

TABLE V

POLYSACCHARIDE PRODUCTION BY *L. betulina* WHEN GROWN ON AQUEOUS EXTRACTS OF FRESH LEAVES OR LITTER

Dilutions§	Litter extracts				
	Calluna*	Beech	W. elm	Ash	Hazel
1:0	−	−	−	−	−
1:0 (G)	−	−	−	−	++
1:1	−	−	−	−	−
1:1 (G)	−	−	−	−	+
1:3	++	−	−	−	−
1:3 (G)	++	++	−	+	+
1:7	+	−	−	−	−
1:7 (G)	++	++	+++	+	+
1(1:3):10 C.D. (G)	++	+++	+++	+	++

NOTE: For legend, see Table IV.

TABLE VI

POLYSACCHARIDE PRODUCTION BY *P. versicolor* WHEN GROWN ON AQUEOUS EXTRACTS OF FRESH LEAVES OR LITTER

Dilutions§	Litter extracts				
	Calluna*	Beech	W. elm	Ash	Hazel
1:0	−‖	−	−	−	−
1:0 (G)	−	−	−	−	++
1:1	−	−	−	−	−
1:1 (G)	−	−	−	−	++
1:3	+	−‡	−	−	−‡
1:3 (G)	+	+‡	−	+	+‡
1:7	+	−‡	−	−	−‡
1:7 (G)	++	+‡	+++	++	+
1(1:3):10 C.D. (G)	+++	++	+++	++	++

NOTE: For legend, see Table IV.
‖Abundant polysaccharide production (+++) after incubation for 2 months.

TABLE VII

POLYSACCHARIDE PRODUCTION BY *M. dryophilus* WHEN GROWN ON AQUEOUS EXTRACTS OF FRESH LEAVES OR LITTER

Dilutions§	Litter extracts				
	Calluna*	Beech	W. elm	Ash	Hazel
1:0	+++	−	−	−‖	−‖
1:0 (G)	+++	+++	−	−	+++
1:1	+++	−	−	−	−
1:1 (G)	+++	+++	−	+++	+++
1:3	+++	−	−	+	−
1:3 (G)	+++	+++	−	+++	++
1:7	++	−	−	−	−
1:7 (G)	+++	+++	−	+++	++
1(1:3):1 W. elm (1:3) (G)	−	−		−	−
1(1:3):10 C.D. (G)	+++	++	−	+++	++
1(1:7):10 C.D. (G) (y.e.)¶			+		

NOTE: For legend, see Table IV.
‖Abundant polysaccharide production (+++) after incubation for 2 months.
¶(y.e.): 0.1 ml. of a 0.5% yeast extract solution added as source of growth factors.

Calluna-leaf or calluna-litter extracts.—The fresh leaf and litter extracts (Tables IV to VII and Table III, respectively) of calluna apparently contained sufficient available carbohydrate material to allow polysaccharide synthesis. The leaf extract does not appear to contain any factor preventing polysaccharide synthesis by the fungi under investigation except in the case of *L. betulina* and *P. versicolor*, at low dilutions.

Beech-litter extract.—This extract permitted polysaccharide production by *P. pullulans* (Table IV). However, in the case of *L. betulina* (Table V) and *P. versicolor* (Table VI), at neither high nor low dilution was there production of polysaccharides. They were produced only when glucose was added to a highly diluted extract. It permitted polysaccharide production by *M. dryophilus* provided glucose had been added (Table VII).

Wych-elm-litter extract.—*P. pullulans* produced polysaccharide on elm-litter extract provided glucose had been added (Table IV). It is obvious, on the other hand, that the extract, even when considerably diluted, contains a factor or factors inhibitory to the synthesis of polysaccharide by the other three organisms (Tables V to VII).

Ash-litter extract.—Ash-litter extract, at low dilution, contains an inhibitor of polysaccharide production by all the organisms investigated although *M. dryophilus* was less sensitive to its action than the other fungal species used.

Hazel-litter extract.—Hazel-litter extract does not apparently contain inhibitor to polysaccharide synthesis although it contains insufficient nutrients to allow polysaccharide production.

Discussion

The extracellular polysaccharides of the four fungi examined are very probably heteropolysaccharides, even though the preparations obtained may be formed by a mixture of components, despite the results obtained by electrophoresis experiments. The nitrogen containing material of the polysaccharides is most probably derived from contaminating nucleic acids and proteins. A number of microbial polysaccharides have been reported to contain up to 1% nitrogen, not derived from hexosamines, the removal of which is very difficult and usually involves degradation of the polysaccharides (see 16).

The experiments carried out using aqueous extracts of plant debris as substrates suggest that polysaccharide synthesis by fungi is possible, within certain limits (absence of competition and antibiosis between microorganisms), under nutritional conditions that may resemble those in which the organisms occur in nature. This is, of course, assuming that factors inhibitory to polysaccharide synthesis are absent from the environment medium and that there are sufficient readily available nutrients to permit polysaccharide synthesis. Yet observations have been made, in the present investigation, which show that undiluted extracts of certain litters (ash and hazel) have permitted polysaccharide synthesis by *M. dryophilus* after 2 months' incubation, although no polysaccharide production occurred under the standard conditions of test (incubation for 20 days). Possible explanations of this phenomenon, such as adaptive utilization of leaf polysaccharides for subsequent fungal polysaccharide production or utilization of a factor which prevents polysaccharide synthesis, could be put forward.

It is noteworthy that polysaccharide production by the fungi tested occurred, even though only with diluted extracts in some cases, whether the organisms were grown on leaf or litter extracts of species associated with mor type of humus or on extracts of litter from species giving rise to mull soils. It is difficult to understand from these findings alone why the process of crumb-structure formation seems to be inoperative in soils associated with mor layers, since all the polysaccharides isolated in the course of this investigation have, in varying degrees, the property of aggregating soil particles (Bernier, unpublished results). Under field conditions where mor formation occurs, it may well be that conditions gradually become inimical to polysaccharide

formation, perhaps owing to competition among the microbial species or to progressively increasing disturbance of the nutrition or metabolism of those organisms capable of synthesizing polysaccharides.

Further study to elucidate the nature of the factors controlling the formation and destruction of microbial polysaccharides under natural conditions should throw some light on the little-understood influence of these metabolic products in soil processes.

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THE OXIDATIVE METABOLISM OF RHODOTORULA GRACILIS[1]

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Abstract

The oxidative metabolism of *Rhodotorula gracilis* NRRL Y-1091 was investigated. Data was presented showing the oxidation of glucose, acetate, pyruvate, xylose, D- and L-arabinose, by both glucose- and xylose-grown cells. Gluconate was oxidized by the glucose-grown cells, while ribose was oxidized by the xylose-grown cells. Cell-free extracts of glucose-grown cells oxidized glucose, gluconate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, and ribose-5-phosphate. Pentose phosphate was demonstrated as a product of glucose-6-phosphate oxidation. Resting-cell suspensions were unable to oxidize citrate, but they oxidized the dicarboxylic acids of the TCA cycle. Citrate was detected as a product of the oxidation of acetate, pyruvate, and malate in the presence of fluoroacetate. Cell-free extracts of glucose-grown cells oxidized citrate, isocitrate, and the dicarboxylic acids of the TCA cycle.

Introduction

In a recent report from this laboratory, the effect of certain environmental factors on the growth-rate characteristics of *Rhodotorula gracilis* NRRL Y-1091 were described (22). Since the results of this study indicated that this organism possessed some characteristics desirable for the production of food or fodder yeast, it was considered worth while to obtain additional information on its metabolism.

There have been few reports in the literature concerning the metabolic activities of yeasts other than those in the genus *Saccharomyces*. The oxidative assimilation of various substrates by *Torulopsis utilis* and *Candida albicans* has been investigated by Fink, Krebs, and Lechner (7) and Van Niel and Cohen (26). Nielsen and Nilsson (18) observed that resting cell suspensions of *R. gracilis* oxidized a variety of carbohydrates, but no data was presented demonstrating the oxidation of possible intermediates in the metabolism of these substrates. More recently, pentose-grown "Torula" yeast was reported to be rich in the enzymes of the hexose monophosphate pathway (23). De Ley and Vandamme (6) investigated the metabolism of 2-ketogluconate by a variety of yeasts. No 2-ketogluconokinase activity could be demonstrated in *Rhodotorula glutinis*.

Materials and Methods

Stock cultures of *R. gracilis* NRRL Y-1091 were carried on malt extract – yeast extract agar as previously described (22). The cells to be used for the metabolic studies were grown in shake flasks in the following medium: glucose 16 g., ammonium sulphate 5.3 g., potassium dihydrogen phosphate 1.5 g., magnesium sulphate heptahydrate 1.0 g., sodium chloride 0.5 g., calcium chloride dihydrate 0.3 g., ferric chloride hexahydrate 0.005 g., and yeast

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extract (Difco) 3 g. per liter. This medium was adjusted to pH 5.5. The cells were grown for 12 hours at 32° C., and then harvested by centrifugation and washed three times with distilled water. In order to reduce the endogenous respiration, the cells were suspended in *M*/45 phosphate buffer, pH 6.5, and agitated in shake flasks for 3 hours at 32° C. prior to use in manometric experiments. It was found that this treatment did not affect the over-all physiological activity of the cells.

Oxygen consumption and carbon dioxide evolution were measured with the Warburg respirometer according to the procedures given by Umbreit, Burris, and Stauffer (25). The following protocol was followed in all experiments in which resting-cell suspensions were used: 20 μM. of the substrate, 1.0 ml. *M*/15 phosphate buffer, pH 5.5, 1.0 ml. cell suspension (10 mg. dry weight), 0.2 ml. 20% KOH in the center well, and sufficient water to bring the total liquid volume in the Warburg vessel to 3.2 ml. The gas phase was air, and a temperature of 30° C. was used in all experiments. Initial studies demonstrated that when the endogenous oxygen uptake was subtracted from the total oxygen uptake, reproducible data for substrate oxidation was obtained. Therefore, all the manometric data reported are net values.

Cell-free extracts were prepared according to the method of Lamanna and Mallette (13). The following system produced active extracts: 22 g. wet-packed cells, 40 g. "superbrite"* glass beads, 5 ml. 0.01 *M* nicotinamide to inhibit diphosphopyridine nucleosidase (10), 5 ml. 0.01 *M* tris(hydroxylmethyl)-aminomethane buffer, pH 7.2, and distilled water to a total volume of 50 ml. This suspension was added to a prechilled micro-Waring blendor jar which had a copper jacket soldered onto the base. This jacket was filled with ice and salt. Water was allowed to trickle over the ice to provide rapid heat transfer. The contents were ground for 45 minutes at 0°– 5° C. and centrifuged at 0°– 5° C. The supernatant was used immediately.

The phosphate esters of the sugars were determined by the paper chromatographic procedure of Cohen and McNair-Scott (5). Pentose was determined by the method of Meijbaum (16), and citrate by the procedure of Saffran and Denstedt (20).

Results and Discussion

As preliminary experiments demonstrated that *R. gracilis* was unable to ferment glucose to either ethyl alcohol or lactic acid, experiments were performed to determine the ability of glucose-grown and pentose-grown cells to oxidize substrates related to the oxidative pathways of carbohydrate metabolism. As shown in Table I, resting cell suspensions grown in the glucose medium oxidized glucose, acetate, and pyruvate rapidly, and gluconate at a much slower rate, while 2-ketogluconate was not oxidized to any significant extent. D-Xylose was oxidized at a substantial rate after a period of adaptation. This adaptation period was more pronounced with D- and L-arabinose, while D-ribose was not oxidized.

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When the organism was adapted to grow on the medium in which 2% D-xylose was the carbon source instead of glucose, such resting cells oxidized D-xylose, glucose, and acetate rapidly, while pyruvate and D- and L-arabinose and D-ribose were slowly oxidized after long induction periods. Gluconate was not oxidized to any appreciable extent by the xylose-adapted cells. Typical results are presented in Table I.

TABLE I

THE NET OXIDATION OF VARIOUS SUBSTRATES BY GLUCOSE-GROWN AND XYLOSE-GROWN CELLS OF *R. gracilis*

Substrate	μl. O_2/300 min.	
	Glucose cells	Xylose cells
Glucose	760	400
Gluconate	120	0
2-Ketogluconate	15	—
Acetate	490	430
Pyruvate	680	215
D-Xylose	690	530
D-Arabinose	210	65
L-Arabinose	145	55
D-Ribose	0	20

In a study of the effects of inhibitors of glycolytic enzymes on glucose oxidation by resting cells, 0.02 *M* fluoride and 0.002 *M* iodoacetate caused 6 and 43% inhibition, respectively. Although this inhibition by fluoride was not significant in comparison with that observed with *Saccharomyces cerevisiae* (19), it cannot be concluded that enolase is not present in the cell. It is quite likely that cellular permeability barriers may have prevented this inhibitor from reaching the active site of enolase within the cell. The observed inhibition of glucose oxidation by iodoacetate does suggest that sulphydryl enzymes such as glyceraldehyde-3-phosphate dehydrogenase may play a role in glucose metabolism by this organism (21).

A concentration of 0.005 *M* arsenite inhibited glucose and pyruvate oxidation 25 and 29%, respectively. Although these values are not as great as those reported for arsenite inhibition of glucose and pyruvate oxidation by *S. cerevisiae*, the comparable extent of inhibition of these substrates suggests that the pyruvate and 2-ketogluconokinase oxidation systems were affected. Such results have been reported by Hockenhull *et al.* for *Streptomyces griseus* (11), and *Penicillium chrysogenum* (12).

In order to obtain further information on the oxidation of intermediates in glucose and pentose metabolism, additional experiments were performed using cell-free extracts. The results presented in Table II show that glucose and gluconate were oxidized by the extracts of glucose-grown cells in the presence of adenosine triphosphate (ATP), triphosphopyridenucleotide (TPN), and magnesium ions (Mg^{++}), while glucose-6-phosphate was oxidized in the presence of TPN and Mg^{++}. Although ribose-5-phosphate was oxidized slowly

without TPN, the addition of this coenzyme resulted in a much greater rate of oxidation. No additional coenzymes were required for the oxidation of either fructose-6-phosphate or fructose-1,6-diphosphate. However, the latter compound was oxidized at a very slow rate.

TABLE II

THE OXIDATION OF GLUCOSE, GLUCONATE, AND PHOSPHORYLATED INTERMEDIARY COMPOUNDS BY CELL-FREE EXTRACTS OF GLUCOSE-GROWN *R. gracilis*

Subtrate	Additions	μl. O_2/120 min.*
Glucose	15 μM. ATP, 15 μM. $MgCl_2$, 1 μM. TPN	115
Gluconate	15 μM. ATP, 15 μM. $MgCl_2$, 1 μM. TPN	68
Glucose-6-phosphate	15 μM. $MgCl_2$, 1 μM. TPN	104
Fructose-6-phosphate	—	89
Fructose-1,6-diphosphate	—	33
Ribose-5-phosphate	1 μM. TPN	66

NOTE: The Warburg vessels contained 10 μM. substrate, 1 ml. 0.05 *M* tris(hydroxylmethyl)aminomethane buffer, pH 7.2, 1 ml. of extract, 10^{-5} *M* methylene blue, 1 μM. DPN, 0.2 ml. 20% KOH in the center well, and water to a total volume of 3.2 ml.
*Endogenous value subtracted: 19 μl./120 min.

A quantity of pentose (2 μM.) over and above the endogenous value, was detected as a product of glucose-6-phosphate oxidation by the Meijbaum procedure. This was confirmed by the detection of ribose-5-phosphate by paper chromatography. These results suggest that glucose-6-phosphate oxidation proceeds through 6-phosphogluconate to pentose phosphate. However, it does not rule out the possibility of glucose-6-phosphate also being oxidized to 6-phosphogluconate, and the latter compound converted to pyruvate and glyceraldehyde-3-phosphate as with *Pseudomonas saccharophila* (15).

In general, these observations indicate that some of the enzymes of both the Embden–Meyerhof and hexose monophosphate pathways are present in glucose-grown cells of *R. gracilis*. However, further investigation will be required before the quantitative significance of these pathways in the metabolism of glucose by this organism can be determined.

The observation that both glucose-grown and xylose-grown cells oxidized acetate and pyruvate suggested further investigation of the metabolism of compounds related to the tricarboxylic acid cycle. Resting-cell suspensions which had been grown in the glucose medium were unable to oxidize citrate, but were capable of oxidizing acetate, pyruvate, α-ketoglutarate, succinate, fumarate, and malate. A comparison of the observed oxygen consumption and carbon dioxide evolution with the amounts theoretically required for complete oxidation is presented in Table III. These substrates were oxidized to a lesser extent than complete oxidation to carbon dioxide and water, as has been commonly observed, and this suggests that these compounds were oxidatively assimilated into cellular material (4).

TABLE III

THE NET OXYGEN CONSUMPTION AND CARBON DIOXIDE EVOLUTION BY *R. gracilis* IN THE PRESENCE OF VARIOUS SUBSTRATES

	Glucose	Acetate	Pyruvate	α-Keto-glutarate	Succinate	Fumarate	Malate
Corrected μl. O_2*	661	428	624	626	448	477	439
Moles O_2 per mole substrate	1.48	0.96	1.39	1.40	1.00	1.06	0.98
Corrected μl. CO_2*	722	424	868	1094	642	904	842
Moles CO_2 per mole substrate	1.61	0.95	1.94	2.44	1.43	2.02	1.88
R. Q. observed	1.09	0.99	1.40	1.74	1.43	1.91	1.92
Theoretical moles O_2 per mole substrate†	6.0	2.0	2.5	4.0	3.5	3.0	3.0
Theoretical moles CO_2 per mole substrate†	6.0	2.0	3.0	5.0	4.0	4.0	4.0
R. Q. theoretical	1.0	1.0	1.2	1.25	1.14	1.33	1.33

*Corrected to 760 mm. and 0° C.
†Number of moles for complete oxidation.

To obtain further information on oxidative assimilation, 2,4-dinitrophenol (2,4-DNP) was employed as an inhibitor. As shown in Table IV, glucose was not oxidized to completion in the presence of 5×10^{-4} or 1×10^{-3} *M* 2,4-DNP, but oxygen consumption was stimulated. A concentration of 5×10^{-3} *M* 2,4-DNP markedly inhibited both respiration and assimilation. In studies with other organisms, comparable conditions and concentrations of 2,4-DNP caused complete inhibition of the oxidative assimilation of glucose (3, 4).

In contrast to glucose, acetate was completely oxidized in the presence of 5×10^{-4} *M* 2,4-DNP. Similar effects have been reported for *Pseudomonas calco-acetica* by Clifton (3), *S. cerevisiae* by Pickett and Clifton (19), and *Escherichia coli* by Goucher *et al.* (9).

Pyruvate oxidation was stimulated by this same concentration of 2,4-DNP, but the total oxygen uptake was less than the theoretical amount for complete oxidation. In the case of α-ketoglutarate, succinate, fumarate, and malate, the addition of 1×10^{-4} *M* 2,4-DNP resulted in a substantial increase in total oxygen uptake. In all cases, however, the amounts were less than those required for complete oxidation. When the concentration of 2,4-DNP was

TABLE IV

THE EFFECT OF 2,4-DINITROPHENOL ON THE NET OXIDATION OF VARIOUS SUBSTRATES BY *R. gracilis*

Substrate*	Molar concn. of 2,4-DNP	Corrected μl. O_2 consumed*	Moles O_2 per mole of substrate	% change over control	% of theoretical O_2 uptake for complete oxidation
Glucose	—	690	1.54	—	26
	5×10^{-4}	872	1.94	+26	32
	1×10^{-3}	1092	2.44	+58	41
	5×10^{-3}	57	0.13	−92	2
Acetate	—	420	0.94	—	47
	5×10^{-4}	917	2.05	+118	103
Pyruvate	—	518	1.16	—	46
	5×10^{-4}	779	1.74	+50	70
α-Ketoglutarate	—	649	1.45	—	36
	1×10^{-4}	1002	2.24	+54	56
	5×10^{-4}	47	0.11	−93	4
Succinate	—	518	1.16	—	33
	1×10^{-4}	936	2.09	+80	60
	5×10^{-4}	263	0.59	−44	17
Fumarate	—	500	1.12	—	37
	1×10^{-4}	868	1.94	+73	65
	5×10^{-4}	0	0.00	−100	0
Malate	—	427	0.95	—	32
	1×10^{-4}	901	2.01	+112	67
	5×10^{-4}	113	0.25	−74	8

*Corrected to 760 mm. and 0° C.

increased to 5×10^{-4} *M*, both respiration and assimilation were markedly retarded. Berstein (2) noted a similar effect of this inhibitor on the oxidation of dicarboxylic acids by *P. saccharophila*.

From these results, it would appear that the reactions involved in the oxidative assimilation of glucose, pyruvate, and the dicarboxylic acids by *R. gracilis* are quite complex as oxygen uptake was either stimulated or suppressed, depending on the concentration of 2,4-DNP. It is possible that some of the individual reactions involved in these assimilations are insensitive to this inhibitor, and do not involve oxidative phosphorylation at the mitochondrial level.

The above results suggested that the effects of inhibitors which affect specific enzymes in the TCA cycle, such as malonate and fluoroacetate, should be investigated. A concentration of 0.04 *M* malonate inhibited acetate, pyruvate, α-ketoglutarate, and succinate oxidation 72, 66, 82, and 98%, respectively. This concentration of malonate is somewhat greater than the inhibitory values for succinic dehydrogenase generally reported in the literature (1). However, Mickelson and Schuler (17) found that 0.04 *M* malonate was required to effect a complete inhibition of acetate oxidation by *Ashbya gossypii*. Bernstein (2) reported that 0.05 *M* malonate caused 71% inhibition of succinate oxidation by *P. saccharophila*, while in the case of *Micrococcus pyogenes* var. *aureus*, Stedman and Kravits (24) observed that 0.033 *M* malonate inhibited acetate and pyruvate oxidation 32 and 11%, respectively.

A concentration of 0.002 *M* fluoroacetate inhibited acetate, pyruvate, and malate oxidation 34, 74, and 99%, respectively. When 20 μM. of each of these substrates was oxidized by resting cells in the presence 0.001 *M* fluoroacetate, 5, 12, and 15 μM. of citrate were formed from acetate, pyruvate, and malate, respectively, after subtraction of the endogenous values.

It appears that the acetate oxidation system of *R. gracilis* is relatively insensitive to fluoroacetate poisoning in comparison with the pyruvate and malate oxidation systems. A similar insensitivity of acetate oxidation to poisoning by this inhibitor has been observed in *Corynebacterium creatinovorans* by Fukui and Van Demark (8).

Since intact cells of *R. gracilis* did not oxidize citrate, cell-free extracts were prepared to determine if this inability to oxidize tricarboxylic acids resulted from cellular impermeability to these substrates as has been reported for other microorganisms (1). The oxidation of the acids of the TCA cycle by cell-free extracts is shown in Table V. All of the acids were oxidized without any period of adaptation. Citrate and isocitrate were oxidized in the presence of TPN and Mn^{++} while in the case of α-ketoglutarate, the addition of coenzyme A (Co A) resulted in a marked increase in the rate of oxidation. This latter observation suggests that succinyl Co A may be an intermediate in α-ketoglutarate oxidation as in *Azotobacter vinelandii* (14).

The oxidation of citrate, isocitrate, and the dicarboxylic acids of the TCA cycle by cell-free extracts of glucose-grown cells without any adaptive lag phase demonstrates the constitutive nature of the enzymes involved. However, the data does not exclude the simultaneous functioning of a dicarboxylic

acid cycle (1). From a comparison of the results obtained with intact cells, cell-free extracts, and inhibitors such as malonate and fluoroacetate with similar results obtained with other organisms for which evidence in favor of a TCA cycle has been presented, it is reasonable to presume that the di- and tri-carboxylic acids are oxidized in the sequence of this pathway, and that the TCA cycle is the more important pathway of terminal respiration in this organism.

TABLE V

THE NET OXIDATION OF THE ACIDS OF THE TRICARBOXYLIC ACID CYCLE BY CELL-FREE EXTRACTS OF *R. gracilis*

Substrate	Additions	μl. O_2/180 min.*
Citrate	1 μM. TPN, 10 μM. $MnCl_2$	152
Isocitrate	1 μM. TPN, 10 μM. $MnCl_2$	155
α-Ketoglutarate	1 μM. coenzyme A	188
Succinate		180
Fumarate		163
Malate		115
Oxalacetate		101

NOTE: All Warburg vessels contained 10 μM. of substrate, $M/15$ phosphate buffer, pH 7.2, 1 ml. of extract, 10^{-5} M methylene blue, 1 μM. DPN, KOH in the center well, and water to a total volume of 3.2 ml.

*Endogenous value subtracted: 46 μl./180 min.

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LACTOBACILLI IN EDMONTON DAIRY PRODUCTS[1]

D. A. Hill and H. R. Thornton

Abstract

Population densities of lactobacilli in 11 market milks and nine churning creams were estimated by the use of selective and enrichment techniques. The incidence of these organisms was low in the milks and only slightly higher in the creams. Circumstantial evidence suggests that non-sterile utensils are the major source during milk production. There was no indication that lactobacilli are important in these two dairy products in this area. The counts of lactobacilli were unexpectedly low during the curing and storage of two of the five Cheddar cheese under scrutiny and these organisms were very unevenly distributed throughout the cheese. The relation of lactobacilli to cheese grade was not revealed in this study. A total of 136 isolates from the quantitative plates were classified according to the scheme of Davis and all were considered to be *Lactobacillus casei*. Of these, 114 cultures fell into one or other of Davis's *L. casei* varieties A, B, and C, while 22 isolates were not classifiable as to variety. The nebulosity of the status of *L. plantarum* as distinct from *L. casei* is discussed.

Introduction

Although the voluminous literature pertaining to the lactobacilli attests to their importance, there have been no studies of their occurrence or importance in Alberta dairy products. The bacteriology of milk in a cold climate can be expected to differ somewhat from that in areas climatically more temperate and this has been shown to be true as regards the lactic streptococci (6) and the psychrophiles (4). An attempt is made herein to determine the status of the lactobacilli in some of the dairy products of this district.

Methods

The milks, except milk number 8, were taken from pooled lots in local milk plants; the churning creams, again with the exception of cream number 8, were from pooled lots in a local creamery. The Cheddar cheese were manufactured during student classes in the Department laboratory, from raw milk in the case of cheese number 1 and from pasteurized milk otherwise. They were stored at 50° F. for 12 weeks and 45° F. thereafter. The cheese were sampled individually by means of sterile triers. From each sample 1 g. of cheese was weighed, ground in a sterile mortar with a portion of a 99 ml. quantity of warm 2% sodium citrate solution (5), and rinsed into a sterile bottle using the remaining citrate solution. An aliquot of this mixture, after further serial dilution when necessary, served as inoculum for the enrichment medium.

Direct plating of milks and creams was tried with seven media. Representative colonies were fished into litmus milk, incubated, and examined macroscopically and microscopically.

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Contribution from the Department of Dairying, University of Alberta, Edmonton, Alberta, Canada. Financial assistance for this research was provided by the Canada Department of Agriculture, Ottawa.

In the enrichment technique the milks, creams, and triturated cheese were serially diluted in dilution blanks prepared according to "Standard methods for the examination of dairy products" (1) and portions of the dilution mixtures were seeded into tubes of enrichment medium.

Enrichment media consisted of reconstituted Bacto skim milk, and of various modifications involving pH adjustment and the incorporation of sodium lactate and sodium propionate in varying combinations. Another enrichment medium was the Briggs–Sharpe thallous acetate broth (2, 7) with the neopeptone replaced by tryptose (hereinafter referred to as thallous acetate broth).

In the early stages of the study the plating medium was either a modified V-8 agar or Rogosa agar and frequently both. Later, agar was added to the thallous acetate broth for plating purposes and the poured plates after hardening were overlaid with a layer of the same medium.

The inoculated and incubated enrichment cultures were either plated or inoculated into litmus milk or both. If plated, colonies were fished into litmus milk.

Three temperature–time combinations were used throughout for incubation, viz., 45° C.—3 days, 35° C.—5 days, and 28° C.—7 to 14 days.

A culture was assumed to belong to the genus *Lactobacillus* if it contained Gram-positive, non-motile, non-sporing rods which failed to liquefy gelatin or produce catalase but reduced and coagulated litmus milk or glucose yeast-extract chalk litmus milk.

For classification, 136 isolates were chosen from many hundreds of available cultures. The choice was not a random one, as an endeavor was purposely made to select as many different types of lactobacilli as possible and representatives were included from the pooled raw milks intended for pasteurization, the churning creams, and the cheese.

The isolates were twice purified on thallous acetate agar and then carried in stab culture in Bacto microassay culture agar. They were prepared for testing by transfer into litmus milk and then into thallous acetate broth, from which they were plated on thallous acetate agar. Colonies were fished into litmus milk, and then transferred into thallous acetate broth, and transferred again into thallous acetate broth, which supplied the inoculum for the test media.

The classification scheme proposed by Davis (3) was followed because of its apparent simplicity, and his testing procedures were meticulously adhered to.

Results

Direct Plating

Attempts to isolate lactobacilli from milk by direct plating were almost completely unsuccessful. Of 420 isolates fished from the pour plates, three were lactobacilli and these were from one milk only, two from plates incubated at 35° C., and one from a plate incubated at 28° C.

Enrichment Technique

No success attended efforts to isolate lactobacilli when plain reconstituted skim milk was the enrichment medium, because of lack of selective action.

The modified skim milks had greater selective properties and isolation of lactobacilli was effected with some difficulty. The thallous acetate broth was very highly selective and yielded nothing but lactobacilli within the incubation times cited.

As seen in Table I, the raw milks contained few lactobacilli as measured by the thallous acetate broth enrichment technique. None of the 11 milks had a count as high as 10,000 lactobacilli per milliliter, no lactobacilli were isolated from two of the milks, and only two milks yielded any lactobacilli at 45° C.

All of the churning creams contained lactobacilli and the tendency for their occurrence in greater numbers than in the milks is apparent from Table II. This was expected because in general these creams are produced with less care and are stored for much longer periods on the farms than are the market milks. Nevertheless, the small number in the creams is noteworthy.

TABLE I

LOGARITHMS OF THE COUNTS OF LACTOBACILLI PER ML. OF MILK

Milk	Month	Incubation at:		
		45° C.	35° C.	28° C.
4	Aug.	1–2*	1–2	Nil
5	Sept.	Nil	1–2	Nil
6	Sept.	Nil	Nil	Nil
7	Sept.	Nil	Nil	1–2
8	Oct.	Nil	Nil	Nil
9	Nov.	1–2	1–2	1–2
16	Feb.	Nil	1–2	1–2
17	Feb.	Nil	Nil	3–4
35	May	Nil	3–4	3–4
40	May	Nil	1–2	2–3
42	June	Nil	3–4	2–3

*Greater than 10^1 and less than 10^2.

TABLE II

LOGARITHMS OF THE COUNTS OF LACTOBACILLI PER ML. OF CHURNING CREAM

Cream	Month	Incubation at:		
		45° C.	35° C.	28° C.
8	Oct.	Nil	1–2	Nil
10	Nov.	Nil	1–2	Nil
11	Nov.	1–2*	1–2	1–2
12	Nov.	2–3	2–3	2–3
13	Dec.	2–3	1–2	Nil
14	Jan.	Nil	1–2	Nil
15	Jan.	1–2	2–3	Nil
39	May	Nil	3–4	3–4
41	May	Nil	4–5	2–3

*Greater than 10^1 and less than 10^2.

Attention is called to milk number 8 and cream number 8, both of which came from a farm producing churning cream. The cream was separated from milk obtained by machine at an evening milking and was held overnight at 46° F. The milk was collected the following morning by hand milking into open sterile utensils and was heavily contaminated with manure and, particularly, soil.

The lactobacilli in the cheese increased from an initial low number to a maximum in about 12 weeks (Table III). In three of the five cheese the maximum

TABLE III

LOGARITHMS OF THE COUNTS OF LACTOBACILLI PER GRAM OF CHEESE

	Cheese				
Weeks cured	1	2	3	4	5
3	4–5*	4–5	4–5	Nil	2–3
6	6–7	6–7	5–6	Nil	4–5
9	7–8	7–8	7–8	5–6	Nil
12	8–9	8–9	8–9	Nil	5–6
18	6–7	6–7	4–5	5–6	5–6
24	6–7	6–7	5–6	5–6	5–6
44	6–7	7–8	7–8		

*Greater than 10^4 and less than 10^5.

TABLE IV

NUMBER AND SOURCE OF CULTURES REACTING POSITIVELY TO 26 PHYSIOLOGICAL TESTS

Test	Milk	Cream	Cheese	Total
Fermentation of raffinose	0	0	0	0
mannitol	27	30	69	126
sorbitol	17	19	32	68
salicin	24	30	68	122
rhamnose	0	7	1	8
lactose	29	36	70	135
melibiose	2	4	1	7
xylose	0	0	0	0
arabinose	0	0	0	0
mannose	29	36	71	136
melezitose	22	27	68	117
Gas from glucose	0	0	0	0
Ammonia from arginine	0	0	1	1
Hydrolysis of sodium hippurate	26	30	69	125
aesculin	29	36	71	136
Acetoin from glucose	19	23	36	78
Tolerance of 0.3% phenol	28	33	71	132
0.4% phenol	25	31	68	124
4.0% sodium chloride	29	36	71	136
8.0% sodium chloride	21	28	48	97
10.0% bile	23	34	53	110
Growth at 15° C.	29	35	71	135
45° C.	3	5	1	9
Gelatin liquefaction	0	0	0	0
Catalase production	0	0	0	0
Average titratable acidity 3–6 ml.	29	36	71	136
Total cultures tested	29	36	71	136

TABLE V

NUMBER AND SOURCE OF CULTURES FALLING INTO THE DAVIS VARIETAL CLASSIFICATION OF *L. casei*

Variety	Milk	Cream	Cheese	Total
A	0	5	1	6
B	18	13	31	62
C	5	7	34	46
Not classifiable as to variety	6	11	5	22
Total	29	36	71	136

count was over 100,000,000. Two of the cheese yielded much smaller numbers. Although the cheese varied in their official scores, it was impossible to correlate the scores and counts of lactobacilli with so few samples.

Classification

Table IV summarizes the reactions of the 136 isolates to 26 physiological tests. Neither motility nor sporeformation was observed at any time, differentiation by morphological criteria was not possible, and no catalase-positive isolate was suspected otherwise of being a lactobacillus. All cultures are considered to be *L. casei*, as the Davis scheme does not clearly separate this species from *L. plantarum*. In Table V, 114 of the cultures can be seen to fall into Davis's *L. casei* varieties A, B, and C, while the remaining 22 isolates are not classifiable as to variety.

Discussion

No brief is held for the accuracy of this method of enumerating lactobacilli in dairy products. The ordinary plate count of bacteria in milk is a very gross estimation lacking in precision. This condition is aggravated when resort is made to selective and enrichment techniques and is again magnified by the heterogeneous distribution of lactobacilli in cheese. Nevertheless, the method was sufficiently sensitive to demonstrate quite clearly the very low incidence of lactobacilli in the market milks and churning creams of this area and no evidence was forthcoming that this bacterial group is important in these products.

Twenty-five years ago lactic acid streptococci and lactobacilli were readily isolated from Edmonton milks by simple methods, even by direct plating or by mere incubation at 45° C. Shortly thereafter the same techniques were not attended by success. The only known widespread change which has taken place in milk production methods since that time is a new emphasis on utensil sterilization. The low incidence of lactobacilli in the milks of the present study, the slightly higher number encountered in the churning creams, and the results with milk number 8 and cream number 8 are all circumstantial evidence that milk-production methods embracing the sterilization of utensils result in milks low in lactobacilli in this area.

The choice of the cheese sample size is always an arbitrary one. More information on bacterial distribution within the cheese is gained from a series

of small samples than from an aliquot of a large sample. Results by the latter technique are of limited value and frequently quite misleading, although the counts may be less variable. The distribution heterogeneity in the cheese studied is impressive but there is no reason to assume the isolates to be non-representative because of the sample size.

The relation of the lactobacilli to the cheese-ripening process and the grade of the cheese was not made apparent by this study. Lactobacilli are not included in the Cheddar cheese starters in this province but the unusual care required for the manufacture of high quality cheese in this district has long been recognized. What bearing the lactobacilli may have on this problem is not known.

Despite efforts to include representatives with varied characteristics, the uniformity of the 136 isolates is striking, indeed. It should be noted, however, that the isolation and selection techniques automatically eliminated any organisms failing to acidify litmus milk. The majority of the cultures fall into Davis's *L. casei* varieties A, B, and C but his proposed segregation lacks definitiveness and it has not been made apparent what benefits derive from such an arbitrary, detailed, and possibly artificial separation.

The taxonomic confusion surrounding the genus *Lactobacillus* has been clearly brought out by a number of investigators and is emphasized in the review by Wheater (9). There is little agreement as to the identity of *L. plantarum*, and Miss Wheater's proposal that it be distinguished from *L. casei* by its action on melibiose awaits general acceptance. It is interesting that, in the present investigation, no culture capable of growth at 45° C. fermented rhamnose (8).

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MORPHOLOGICAL COMPARISON OF SOME ARTHROBACTER SPECIES[1]

VERONICA SUNDMAN

Abstract

Six *Arthrobacter* species and two unidentified organisms of the same genus have been studied morphologically. *A. terregens* differs from the other seven strains as regards the mode of segmentation into coccoids. Two typical cell configurations of the myceloid stage are described. The chromogenic strains show less branched forms than the non-chromogenic strains.

Introduction

Different *Arthrobacter* species have repeatedly been subjected to morphological studies (2, 6, 7, 8) since Conn and Dimmick (4) described the type species *A. globiformis*. However there is no uniform conception as regards the life cycle exhibited by the different species, the mode of initial development of growing coccoids, the significance of the large coccoids, etc. Further there is no information on the morphological variations within the genus.

Some *Arthrobacter* strains were included in a larger comparative study in this laboratory on the morphology of the branched bacteria. They were found to possess some common characteristics not previously reported and some morphological differences big enough to allow a separation on this basis. The observations are reported in this paper because they might be of some value in mapping out this relatively new genus.

Material and Methods

The organisms studied were six named *Arthrobacter* strains received from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa*—*A. aurescens*, *A. citreus*, *A. globiformis*, *A. pascens*, *A. ureafaciens*, and *A. terregens*—and two unidentified *Arthrobacter* strains: 2*c* and 3*b* isolated from local soil.

The medium used was ordinary nutrient agar, except for *A. terregens*, which was grown on the following soil extract agar according to personal advice from Lochhead: 1 kg. garden soil is heated in the autoclave for 20 minutes at 15 lb. The mixture is then filtered and brought up to 1 l. To the filtrate is added 0.02% K_2HPO_4, 0.1% yeast extract, and 1.5% agar, pH 6.8.

All cultures were incubated at room temperature (about 18°). The morphology of the strains was observed in preparations made from surface growth on agar plates. The plates were inoculated with small volumes of water suspension of an agar slant old enough to contain coccoids only. The agar surface was entirely moistened by tilting and the excess of inoculum was

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poured off. After suitable incubation times, blocks were cut out of the agar. The young growth stages were studied in contact prints; the growth surface of the agar block was lightly pressed against a clean slide. Later on when visible growth occurred, the contact prints were too thick to allow good observations. It was then necessary to make repeated prints; the same block was repeatedly pressed against the slide, thus leaving fewer and fewer cells on the glass surface. Ordinary smears were also made from the later growth stages. In order to avoid artifacts, the preparations were left unfixed.

The following stains were used: (1) crystal violet in 0.5% or 0.05% water solution for 30 seconds—the latter dilution was used only in some instances when strongly basophilic cells trended to get overstained with the stronger solution; (2) tannic acid – crystal violet cell-wall stain as recommended by Webb (9), omitting the Congo red, which did not in the present case show any advantage as compared with tannic acid – crystal violet only. The stained preparations were photographed unmounted.

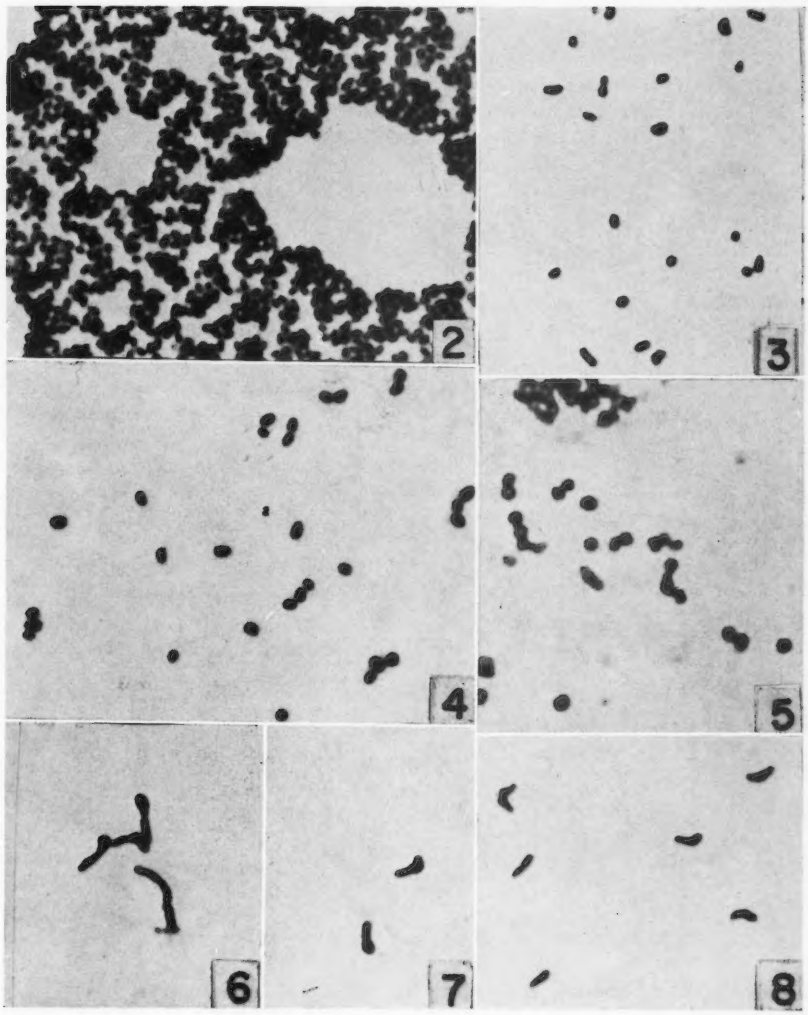
Positive phase-contrast micrographs were made of water-mounted preparations.

Results and Discussion

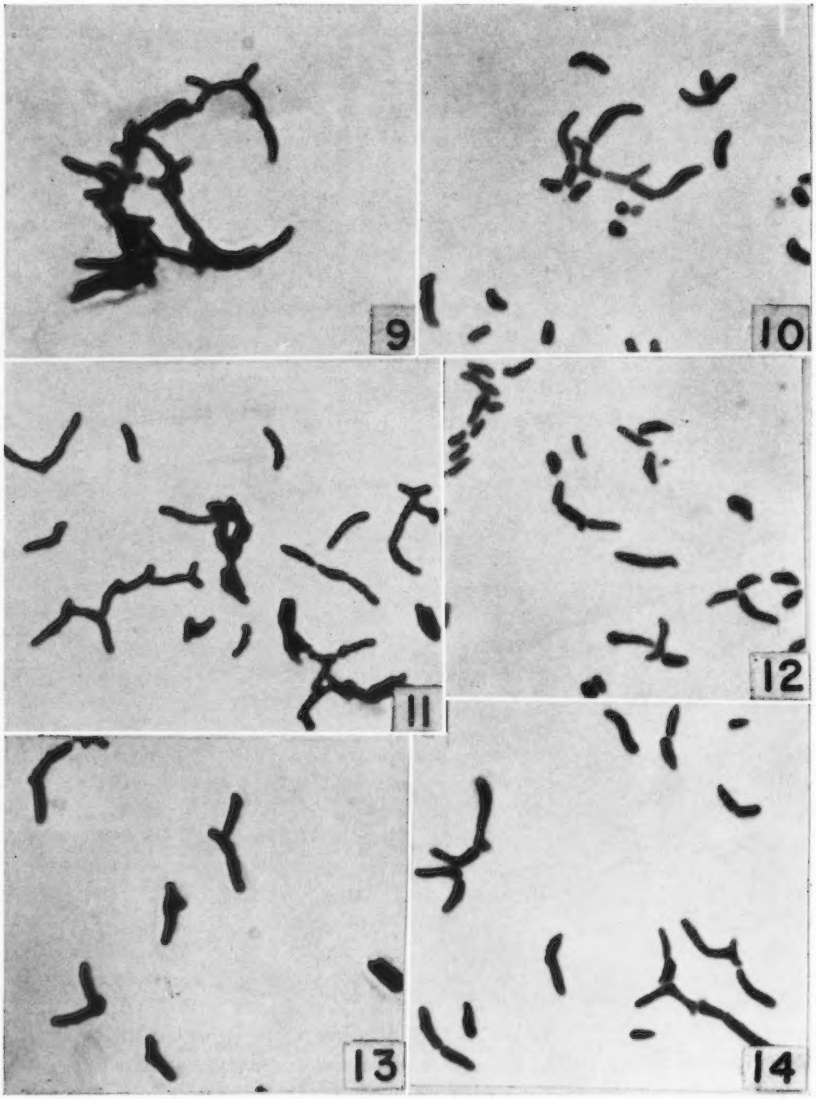
Of the named *Arthrobacter* species studied, *A. terregens* differs from the other five species, being dependent on the "terregens factor" as shown by Lochhead and Burton (6). The inability to liquefy gelatin is a further feature which separates *A. terregens* from the other species of this genus. In a recent description of some "terregens factor" requiring organisms (1), Burton states that they change "from rod forms of the diphteroid type in young cultures to coccoid forms in older cultures". This general scheme fits all cultures studied, but *A. terregens* is nevertheless morphologically distinctly different from the other strains.

The typical morphology of *Arthrobacter* at different stages of growth is seen in Figs. 2–20. The general appearance is the same as described for *A. oxydans* (8) and *A. citreus* (7). The coccoids* of old cultures group in stained smears like micrococci and trend to aggregate (Fig. 2). In thin phase-contrast preparations they are often arranged in pairs with a tendency to typical angular figuration between round and cuneiform cells (Figs. 3, 4). When transferred to fresh medium the coccoids, frequently remaining attached, germinate as described by Sguros (8) to a myceloid stage (Figs. 6–18). Branches are not uncommon at this stage of development (Figs. 9–18). It is doubtful whether with these cultures the development of branches can be linked with any deficiency of the medium as is shown by Chaplin and Lochhead (3) to be the case with a B_{12}-requiring *Arthrobacter* strain; the branches were frequently found in the most abundantly growing strains of *A. globiformis* and *A. pascens*. The branched myceloids are septate. The budlike branches are formed adjacent to septa (Figs. 15, 16). Structures named "opposite and adjacent

*The round or rounded segments are here termed coccoids. To avoid confusion, the terms 'cystite' and 'arthrospore', which are evidently used discrepantly in the literature concerning *Arthrobacter*, are avoided.



FIGS. 2 to 8. Coccoids of *Arthrobacter* species. × 2500
FIG. 2. *A. ureafaciens*, 7 days, crystal-violet stain; micrococcus-like grouping of cells. FIG. 3. Same as Fig. 2, phase contrast; note angular configuration of the coccoid pair at lower right. FIG. 4. Strain 2*c*, 4 days, phase contrast. FIG. 5. *A. pascens*, 7 days, cell-wall stain; note the coccoid dividing by a transverse septum. FIG. 6. Strain 3*b*, 10 hours, crystal-violet stain; germinating coccoids, one with two "germ tubes". FIG. 7. *A. citreus*, 10 hours, crystal-violet stain; germinating coccoids. FIG. 8. Strain 2*b*, 10 hours, crystal-violet stain; germinating coccoids; note the pair at upper right remaining attached during germination.



FIGS. 9 to 14. Species of *Arthrobacter* at the myceloid stage of growth. 2500 ×. 20-hour cultures. Figs. 9, 11, 13, crystal-violet stain; Figs. 10, 12, 14, phase contrast. FIG. 9. *A. globiformis*. FIG .10. *A. pascens*. FIG. 11. Strain *3b*. FIG. 12. *A. ureafaciens*. FIG. 13. *A. citreus*. FIG. 14. *A. aurescens*.

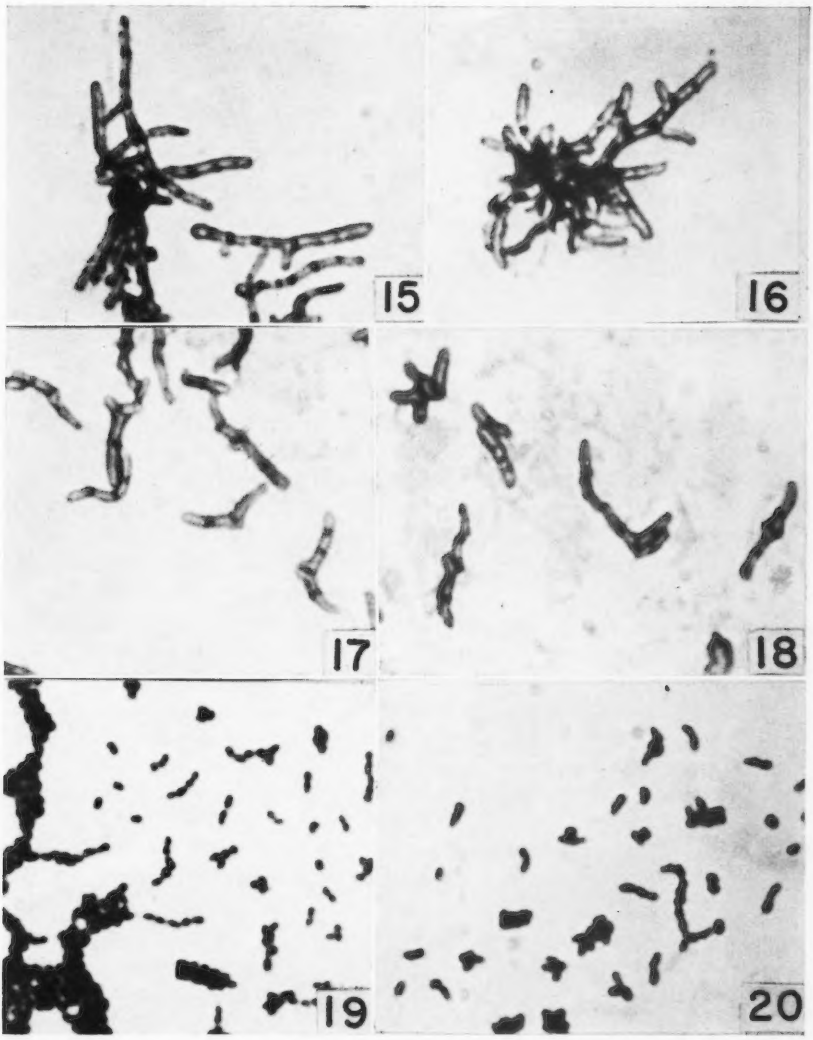
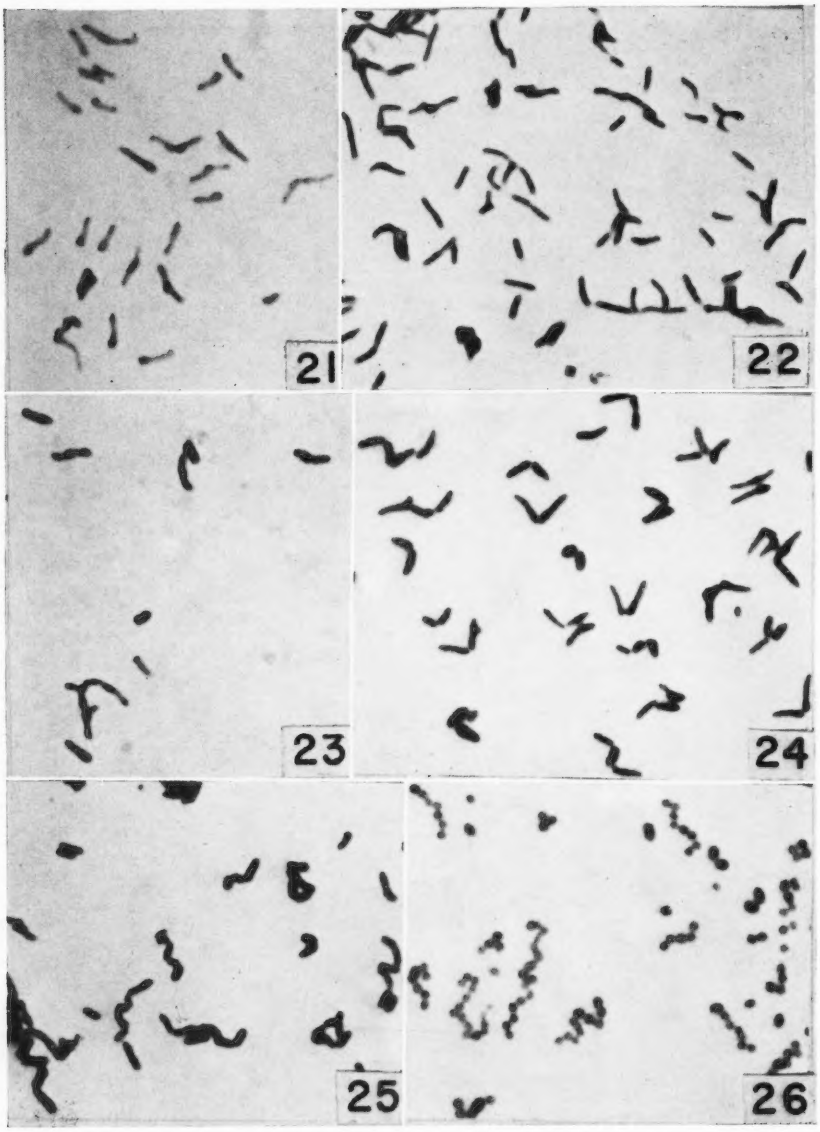


FIG. 15. *A. pascens*, 20 hours, myceloid stage, cell-wall stain, 2500 ×; note position of branches adjacent to septa. FIG. 16. *A. globiformis*, same as Fig. 15. FIG. 17. *A. aurescens*, 20 hours, myceloid stage, cell-wall stain, 2500 ×; "adjacent and opposite angles" (see text). FIG. 18. *A. citreus*, 20 hours, myceloid stage, cell-wall stain, 2500 ×; "opposite parallel angles". FIG. 19. Strain 3*b*, 32 hours segmentation of rods, crystal-violet stain, 2500 ×; the coccoids are preformed before segmentation. FIG. 20. *A. aurescens*, 48 hours, same as in Fig. 19.



FIGS. 21 to 26. *A. terregens* at different stages of growth, 2500 ×.
FIG. 21. Ten hours, germinating coccoids, cell-wall stain. FIG. 22. Twenty-four hours, myceloid stage, crystal-violet stain. FIG. 23. Same as in Fig. 22, cell-wall stain. FIG. 24. Same as in Fig. 22, phase contrast. FIG. 25. Forty-eight hours, segmentation of rods, phase contrast; note zigzag segmentation in short rods. FIG. 26. Mature coccoids, 7 days, phase contrast; note arrangement in zigzag chains.

FIG. 1. Schematic presentation of two configurations seen in *Arthrobacter* cultures at the myceloid stage of growth: *a*, "adjacent parallel angles"; *b*, "opposite parallel angles".

parallel angles" are typical for the myceloid stage. They are schematically drawn in Fig. 1 and clearly seen in cell-wall-stained preparations (Figs. 17, 18).

When stained by basic dyes the "parallel angles" have an appearance like a thicker central node with two slender tubes emerging mostly at an obtuse angle (Fig. 13).

Within 48 hours the rods and myceloids break up into short fragments, which frequently already at the time of segmentation exhibit the same dimensions as the mature resting coccoids (Figs. 19, 20). After segmentation, division of the fragments by a transverse septum is not uncommon (Fig. 5).

Cystites with a different life cycle, as the coccoids described for *A. pascens* by Chaplin (2), have not been observed under the conditions of this work. Coccoids larger than the average were occasionally seen, but they seemed to develop in the manner described here.

The morphology of *A. terregens* at different stages of the life cycle (Figs. 21–26) is in many respects different from the general morphology described above. The most striking difference is the mode of segmentation. In the *A. terregens* strain studied the coccoids are not preformed in the myceloid stage as seems to be the rule for the other strains. The primary stage in segmentation is a breaking up in typically zigzag-arranged short rods (Fig. 25). They later divide to form two coccoids which remain attached after the division to form long streptococcus-like chains where the zigzag formation of the short rods often is recognized (Fig. 26). The mechanism of segmentation reminds one of the course of events in *Nocardia corallina* as described recently by Webb and Clark (10).

Further, the coccoids of *A. terregens* lack the tendency to group in staphylococcus-like large clusters. Even in thick preparations the coccoid chains behave as individuals without any striking affinity to the nearby lying cells. The cell-wall of *A. terregens* is faintly stained by the tannic acid – crystal violet method used. The other strains are very clearly stained. The difference appears by comparing Figs. 21 and 23 with Figs. 15 to 18.

At the myceloid stage the studied strains, except *A. terregens*, show a strong affinity to crystal violet. The cells appear well-stained and sharply defined (Figs. 9, 11, 13). *A. terregens* stains more weakly. The rods are more slender and diffuse, and assume in the early growth phase a pointed appearance (Fig. 22).

Lochhead (5) has pointed out the different distribution of chromogenic and non-chromogenic *Arthrobacter* species in soil, the former being typically rhizosphere inhabitants. This probably depends on more complex nutritional demands in the chromogenic strains. Morphologically the non-chromogenic strains are separated from the chromogenic ones by a stronger tendency to form branches at the myceloid stage. In the non-chromogenic strains studied—*A. globiformis*, *A. pascens*, *2c*, and *3b*—promycel-like structures are frequently seen (Figs. 9–11), whereas in the chromogenic strains—*A. aurescens*, *A. citreus*, *A. ureafaciens*, and *A. terregens*—the branches are budlike and more occasional (Figs. 12, 13, 14, 24).

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TOXICITY OF A UNIALGAL CULTURE OF MICROCYSTIS AERUGINOSA[1]

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Abstract

A unialgal culture of *Microcystis aeruginosa* Kütz. emend. Elenkin, which is toxic when injected intraperitoneally into white mice, has been isolated. It produces a complex of symptoms similar to those which have been described for a number of toxic waterblooms. Two distinct factors seem to be present. One causes death in 4 to 48 hours preceded by symptoms of piloerection and dyspnea. It is detected only with freshly harvested cells. The other causes death within 2 to 3 hours preceded by pallor and convulsions. This second factor is an endotoxin which is detected only when cells become leaky or disintegrate. The presence of the slow-death factor tends to be obscured by the release of the fast-death factor. The concentration of the fast-death factor in the cells varies with the developmental stage of a culture and at maximum can be just as high as that of a very toxic waterbloom.

Introduction

The death of livestock and poultry after drinking water heavily contaminated with growths of blue-green algae has been reported from many parts of the world during the past 75 years (10, 12, 20). Several extensive investigations of this problem have been carried out, but with few exceptions (17, 24) these have depended for their source material on collections of naturally occurring waterblooms. Some collections of waterblooms have proved highly toxic, while others have not. This variability has been attributed to differences in the algal species predominating in different collections (12) and also to the necessity of attaining a critical stage of decomposition (10). The uncertain occurrence of blooms and their variable toxicity have greatly hindered the development of a satisfactory understanding of algal poisonings. Whether the deaths produced by toxic blooms are caused by the algae, by other microorganisms associated with them, or by both, is not clear. Bacterial infections, however, are not the direct cause of death since the toxic principle can be passed through a bacterial filter or autoclaved without loss of potency (17, 25).

One of five species of blue-green algae most suspected of toxicity is *Microcystis aeruginosa* (10). Olson (17) has isolated and grown a toxic strain of this species in unialgal culture. The toxicity to white mice was low and varied greatly from one harvest to the next. No symptoms were described. Gerloff, Fitzgerald, and Skoog (8, 9) have studied the nutrition of a pure culture of *M. aeruginosa* but have not reported on its toxicity. Recently, Thompson, Laing, and Grant (24) have reported the isolation of a strain of

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this species which when grown on mineral solution in either pure or unialgal culture was non-toxic to mice. In this paper we shall describe the isolation of a unialgal culture of *M. aeruginosa* that is toxic to mice, grows well under laboratory conditions, and produces a complex of symptoms similar to those described for a number of different waterblooms. The effects of different treatments and culture conditions on toxicity will also be presented.

Methods

Nomenclature

The genus *Microcystis* has undergone extensive changes in nomenclature in recent years (2, 4). According to Drouet and Daily (4, 5), the organism used in this work should be called *Anacystis cyanea* Drouet and Daily. Because the name *Microcystis* has been used in most of the previous literature on toxicity of blue-green algae, we have retained it and have called the organism *M. aeruginosa* Kütz. emend. Elenkin.

Isolation of Strain NRC-1

A waterbloom containing *M. aeruginosa* as a minor constituent was collected at Little Rideau Lake, Ontario, on September 27, 1954. A single colony of *M. aeruginosa* was transferred to modified Fitzgerald solution (7) and allowed to grow at 21° C. and 400 ft-c. continuous illumination. Single colony transfers were made until the culture was unialgal, as judged by microscopic examination and by tests for growth of green algae using modified Knop's solution, pH 5.5, and modified Gaffron's solution (1), pH 8.0.

A dilute suspension of the unialgal culture was exposed in a thin layer to ultraviolet light (G. E. 15-w. germicidal lamp, 24-in. distance) for 3 minutes. From this, subcultures were inoculated after various exposure times and those that grew were tested for purity by microscopic examination and heavy inoculation into the following media:

1. Difco tryptone glucose extract agar, pH 7.0, 32° C. (aerobes).
2. BBL thioglycolate medium, (an old formulation similar to present-day BBL No. 136) with 1% dextrose and 2% agar added, pH 7.6, 32° C. (anaerobes).
3. Difco potato dextrose extract agar, pH 5.6, 28° C. (molds).

These media were incubated for 2 days in darkness (in later work, 5 to 6 days in light) at the indicated temperatures followed by 6 (in later work, 6 to 10 days) in the laboratory. By repeating the ultraviolet irradiation of the least contaminated subcultures, a culture (designated NRC-1) was eventually obtained that was regarded as pure, and was reported as such in papers read before the Phycological Society of America in 1955 (11) and the Schweizerischen Naturforschenden Gesellschaft in 1956 (26). However, recent work (which will be described in another paper) with different stains, test media, and incubation conditions has indicated that a number of bacterial contaminants are present in this culture.

At first, the culture consisted of large globose colonies with diffluent sheaths, but after many transfers the colonial habit more or less disappeared

and the culture now consists mostly of single and paired cells with occasional small colonies of 10 to 12 cells possessing diffluent sheaths. The cells have pseudovacuoles and are 4 to 5 μ in diameter.

Culture and Harvest

Two culture solutions were used. The first was a modified Fitzgerald solution (7) which differed from the original in having 40 ml. of soil extract and 0.08 ml. of Gaffron's minor element solution* added per liter. The second, called No. 11, was found to promote much better growth. Its composition is as follows:

$NaNO_3$	0.496 g.	$Na_2SiO_3 . 9H_2O$	0.058 g.
K_2HPO_4	0.039 g.	Ferric citrate	0.006 g.
$MgSO_4 . 7H_2O$	0.075 g.	Citric acid	0.006 g.
$CaCl_2 . 2H_2O$	0.036 g.	EDTA	0.001 g.
Na_2CO_3	0.020 g.	Gaffron's minor element solution	0.08 ml.

Demineralized water to 1 liter.

Both culture solutions were prepared by autoclaving the iron separately (18), and adding it after allowing them to stand for 24 hours. The initial and final pH's of cultures in solution No. 11 were 8.0 to 8.5 and 9.0 to 10.0, respectively.

Cultures were grown in temperature-controlled rooms in still flasks, shake flasks, or vertical glass columns of various sizes. The latter were similar to the "tower-type" fermentor described by Martin and Waters (14) but differed in the lack of foam-breaking equipment and in a modification of the sampling tube to permit aseptic handling. The columns were aerated with filtered compressed air which was metered at the inlet. Cultures were illuminated with 30- or 40-watt "white" fluorescent lamps equipped with reflectors. Light intensities were estimated by means of a Weston foot-candle meter which was not color-corrected. Since the columns were illuminated equally from opposite sides, rendering exact integration difficult, intensities were expressed as two times the value obtained for one side. All culture equipment was autoclaved or steam-sterilized and aseptic technique was employed throughout.

The size and age of inocula were not rigidly controlled. Inoculum cultures were grown under conditions similar to those used for experimental purposes. Fairly heavy suspensions (0.5 to 1.0 g. dry wt./l.) were used at a dilution of about one to nine.

*Obtained from Prof. H. Gaffron, Institute of Radiobiology, University of Chicago. Its composition in g./l. is as follows:

H_3BO_3	3.100	KBr	0.119
$MnSO_4 . 4H_2O$	2.230	KI	0.083
$ZnSO_4 . 7H_2O$	0.287	$Cd(NO_3)_2 . 4H_2O$	0.154
$(NH_4)_6Mo_7O_{24} . 4H_2O$	0.088	$NiSO_4(NH_4)_2SO_4 . 6H_2O$	0.198
$Co(NO_3)_2 . 4H_2O$	0.146	$VOSO_4 . 2H_2O$	0.020
$Na_2WO_4 . 2H_2O$	0.033	$Al_2(SO_4)_3K_2SO_4 . 24H_2O$	0.474

Growth was expressed in terms of dry weight of cells per liter. Aliquots of algal suspension were evaporated with gentle warming under a jet of air, dried in an oven overnight at 60° C., cooled in a desiccator, weighed, and a correction for the salts of the solution applied. In one experiment, growth was also estimated by cell counts (average of duplicates) made with a Petroff-Hausser counting chamber.

The cells were precipitated with 10 ml. of 2% aluminum sulphate and 10 ml. of 0.1 *N* hydrochloric acid per liter of culture (9) to permit satisfactory centrifugation (1000 × *g*, 5 to 10 minutes). The supernatants (culture filtrates) were decanted carefully and the packed cells were resuspended in a minimum of distilled water. The culture filtrates were either discarded or evaporated to dryness with gentle warming under a jet of air and extracted with ethanol or methanol (21). The filtered extracts were evaporated, dried, and weighed.

Cell Treatments

Cell suspensions were incubated in darkness at 36° to 37° C. in well-filled serum bottles that were stoppered to restrict the air supply.

Suspensions of cells were frozen rapidly by immersing the containers in a bath of acetone and dry ice (−75° C.). To increase permeability, the cells were rapidly thawed and refrozen two or three times. In one experiment, cells were frozen in this manner, lyophilized, and stored at room temperature for long periods without loss of toxicity.

Cells were disintegrated with a Mickle sonic disintegrator (16) equipped with modified vessels to permit aseptic handling. Using 2 ml. of Chance No. 12 ballotini to 5 ml. of algal suspension for 15 minutes, the temperature rise was 2° to 3° C., and disintegration, as indicated by cell count, was 75% complete.

Bio-assay

The toxicity of the cells was determined by intraperitoneal injection into male white mice of 20- to 30-g. body weight using a standard scale of dosage (20, 40, 80, 160, 320, and 480 mg. dry wt./kg. body weight). The toxicity of the culture filtrates was only determined qualitatively because there was seldom sufficient material to inject at more than one dosage level (160 mg./kg.). The number of mice injected per dose varied from two to six in different experiments. The volume injected was constant for any one experiment, but varied from 0.2 to 0.3 ml. according to the average weight of the mice. Sterilized syringes and needles were used throughout, but samples were prepared in clean unsterilized glassware with unsterilized distilled water after preliminary experiments showed that aseptic precautions were unnecessary. Controls were run to check the toxicity of culture solutions and flocculating reagents. Mice were observed closely for symptoms and survival times during the first 3 to 4 hours after injection and thereafter at intervals for at least 48 hours. Since the dosage–response curve for the factors

involved proved to be very steep in the critical range, the minimum dose required to produce 100% mortality (LD_{100}) was used to assess potency rather than the more precise LD_{50}.

Results and Conclusions

In a preliminary experiment, freshly harvested cells from a 40-day culture grown on modified Fitzgerald solution (19° to 21° C. and 800 to 1000 ft-c.) were injected at a dosage of 550 mg./kg. All the mice sickened and died without convulsions in 24 to 52 hours. Cells from the same culture, incubated for 11 or 23 hours and injected at the same dosage, produced pallor and convulsions followed by death of all mice in 30 to 60 minutes. The culture filtrate was non-toxic at a dosage of 7 mg./kg.

The effects of incubation were re-examined with duplicate 3-day cultures grown on solution No. 11. Similar but not identical results were obtained (Table I). With culture No. 1, the control cells produced slow deaths while the incubated cells produced fast deaths. With culture No. 2, however, the control cells as well as the incubated cells produced fast deaths. Incubation increased the toxicity of the cells from both cultures by a factor of three to four. It also caused the release of large amounts of phycobilin pigments without causing any disorganization of the cells that could be detected under the microscope. The control cells from both cultures produced slow-death *symptoms* at sublethal dosages. Culture filtrate No. 1 was clear and the extract was non-toxic at 240 mg./kg. while culture filtrate No. 2 contained traces of phycobilin pigments and the extract produced fast deaths at 160 mg./kg. Consistent with a loss of toxin into the culture medium, cells from culture No. 2 were less toxic than those from culture No. 1.

The symptoms associated with slow deaths were as follows: after a latent period of 20 to 40 minutes piloerection (hair standing up) was observed, the nose took on a reddish-purple hue, and breathing became somewhat labored (dyspnea). The animals did not eat, some had difficulty in expelling faeces, and most remained quiet and huddled in sickly condition until death ensued.

TABLE I

EFFECTS OF INCUBATION ON TOXICITY

Soln. No. 11, 1.0 l./3-l. shake flask, 160 ft-c., 28±0.1° C., 500 cc./min. aeration, 3 days. Two, male, 20-g. mice/dosage level. Dosage range 40 to 480 mg./kg.

Sample	Culture No. 1		Culture No. 2	
	Max. survival time, hr.	LD_{100}, mg./kg.	Max. survival time, hr.	LD_{100}, mg./kg.
Control cells*	24–48	320†	3.3	480‡
Incubated cells (15 hr.)	2.3	80	2.4	160

*Stored at 5° C., 15 hours.
†Slow deaths also produced at 480 mg./kg.; slow-death symptoms produced by all sublethal doses down to and including 40 mg./kg.
‡Slow-death symptoms produced by 320 mg./kg.

A frequently observed stickiness of the eyelids may have been related to the white secretion from the eyes reported with slow deaths in other animals (6). The color of tail, ears, and capillaries of the abdominal wall indicated that peripheral circulation was normal. The liver was pale, often blotchy, and did not bleed on incision. The intestinal mucosae were dotted with "clots" of algal cells that, under the microscope, appeared to be agglomerated with fatty substances. The heart was normal and still beating when autopsies were done immediately. In a few animals, lung haemorrhage was observed, but mostly the lungs were normal.

The fast-death symptoms were similar to those which have been described for mice and other small animals injected or fed *Microcystis* bloom material (13, 21, 23, 25). There was a latent period of 15 to 30 minutes following the injection during which the mice behaved normally; then convulsive jumps, dragging of the hind legs, and an apparent loss of equilibrium were observed. The animals underwent alternating periods of restlessness and apathy accompanied by an increasing pallor of the tail and ears and a change in eye color from red to light pink. Death was preceded by a brief period of spasmodic breathing. Immediate autopsy revealed a greatly reduced supply of blood in both the peripheral and, to a lesser extent, the visceral circulatory systems. The liver was dark and engorged and bled when incised. The heart, which was still beating, and the lungs were normal.

These incubation experiments suggested that more than one toxic factor was involved. Besides the obvious differences in symptoms, there was the supporting evidence that a higher dosage of cells producing slow deaths did not produce fast deaths, and that a lower dosage of cells producing fast deaths did not produce slow deaths. The slow-death factor (SDF) appeared to be associated with the cells rather than the culture filtrate. The presence of appreciable amounts of the fast-death factor (FDF) completely obscured the presence of SDF. Whether incubation caused the production of new FDF or the release of FDF already present in the cells was not clear. However, the detection of FDF in both the control cells and the culture filtrate of culture No. 2 under conditions which permitted the release of high-molecular-weight phycobilins suggested that FDF was a water-soluble endotoxin that had to be liberated from the cells before it could act.

In subsequent experiments, deaths were classified on the basis of symptoms as well as survival times. Since most fast deaths occurred within 2 or 3 hours, and most slow deaths occurred between 4 and 24 hours, survival for shorter or longer periods than 4 hours was taken as the criterion for classification.

Wheeler, Lackey, and Schott (25) have reported that freezing caused an increase in toxicity of *M. aeruginosa* obtained from blooms and that it caused the toxic substance to go into solution. The effects of freezing were therefore compared with those of incubation. Freshly harvested cells of *M. aeruginosa* NRC-1 were either frozen and thawed five times, incubated for 21 hours, given the freezing treatment followed by the incubation treatment,

or given the incubation treatment followed by the freezing treatment. Freezing caused partial disintegration of the cells without releasing the phycobilins while incubation again caused leakage of these pigments. The control produced fast deaths at the highest dosage tested (320 mg./kg.), some slow deaths at the next lower dosage, and slow-death symptoms at all lower dosages (Table II). Freezing, incubation, or combinations of both caused the cell suspensions to produce only fast deaths and decreased the LD_{100} to the same value in every case. Since the effects of the two treatments were similar rather than additive, each must have brought about the same result, namely, the complete release of the FDF that was present in the cells to begin with. The release of FDF unaccompanied by the release of phycobilins confirmed the fact that these pigments have nothing to do with the toxin (21, 23).

As a further test of the hypothesis that FDF was an endotoxin already present in the cells which incubation and freezing only served to release, the toxicity of mechanically disintegrated cells was compared with that of incubated cells. The control produced no deaths, only slow-death symptoms, at the highest dosage tested (Table III). Disintegration proved to be just

TABLE II

EFFECTS OF INCUBATION AND OF FREEZING ON TOXICITY

Soln. No. 11, 700 ml./1-l. still flask, 150 to 200 ft-c., 21° to 24° C., 15- and 25-day cultures pooled. Four, male, 25-g. mice/dosage level. Dosage range 20 to 320 mg./kg.

Treatment of cells	Effect of injection	LD_{100}, mg./kg.
Control*	Fast death	>320†
	Slow death	>160‡
Incubated (21 hr.)	Fast death	80
Frozen	Fast death	80
Incubated-frozen	Fast death	80
Frozen-incubated	Fast death	80

*Culture solution and culture filtrate containing 100 p.p.m. $Al_2(SO_4)_3$ were non-toxic.
†75% mortality at this dose.
‡50% mortality at this dose; slow-death symptoms produced by all sublethal doses.

TABLE III

EFFECT OF INCUBATION AND OF SONIC DISINTEGRATION ON TOXICITY

Soln. No. 11, 2.7 l./3.5-l. column; 800 ft-c., 27 to 32° C., 1000 cc./min. aeration, 5 days. Four, male, 25- to 30-g. mice/dosage level. Dosage range 20 to 320 mg./kg.

Treatment of cells	Effect of injection	LD_{100}, mg./kg.
Control	Slow-death symptoms*	—
Incubated (18 hr.)	Fast death	160
Disintegrated (75%)	Fast death	160
Incubated–disintegrated	Fast death	160
Disintegrated–incubated	Fast death	160

*At 160 and 320 mg./kg.

as effective as incubation in releasing FDF. Both treatments and combinations thereof resulted in the cells having the same LD_{100}. They also abolished the slow-death symptoms observed with the control at corresponding sublethal dosages. Mechanical disintegration, like freezing, caused no accompanying release of phycobilins into solution.

Aerated cultures of *M. aeruginosa* NRC-1 sometimes ceased to grow and started to foam. After a period of weeks such cultures often became milky white to almost clear in appearance. Microscopic examination showed that the algal cells had become completely lysed. Since the previous experiments had shown that treatments causing cellular breakdown led to the release of FDF, a number of completely lysed cultures, 21 to 31 days old, were pooled and tested for the presence of this factor as follows: a 3-liter aliquot was adjusted from pH 9 to pH 6 with HCl, concentrated to 200 ml. under reduced pressure at 35° to 40° C., then evaporated to dryness and extracted with 95% ethanol in the manner described for culture filtrates. This extract produced fast deaths but contained significant quantities of sodium nitrate which caused pronounced, although transitory, additional symptoms when injected into the mice. The LD_{100} was 200 mg./kg. The sodium nitrate was removed by repeating the extraction process several times with absolute alcohol, but there was a loss of more than half of the toxin. The LD_{100} of the final extract was 25 mg./kg.

A rough calculation was made of the amount of FDF to be expected in the cells before lysis and this was compared with the amount found in the 95% ethanol extract. It indicated a probable recovery of between 50 and 100%. FDF, therefore, withstands the alkaline conditions of the medium for long periods better than was to be expected on the basis of Shelubsky's work (21). She had reported the complete destruction of a factor like FDF by treatment with 0.1 *N* NaOH for 1 hour at 100° C.

Having noted that both leakage and lysis can cause the release of FDF, we carried out two experiments at different times to determine at what developmental stage the cells of an actively growing culture would reach maximum toxicity and also how toxic they would be. Cultures were grown in a large glass column at room temperature. Daily samples were taken for growth and toxicity determinations. Cells were twice-frozen, lyophilized, and stored at room temperature until all samples were ready for the toxin assay. In the second experiment, cell counts were also made and absolute methanol extracts of the culture filtrate prepared and tested for toxicity. The latter tests made it necessary to take very large samples so the experiment had to be terminated after 7 days.

In the two experiments, neither the patterns of growth during the first few days nor the patterns of changing toxicity were quite the same (Fig. 1). In the first experiment, the LD_{100} of the cells reached a minimum on the fourth and fifth days when the dry weight of cells was about 0.8 g./l. Thereafter it increased as the culture began to foam until, by the 14th day, most of the toxin had disappeared. Cell dry weight reached a maximum 2 to 3 days

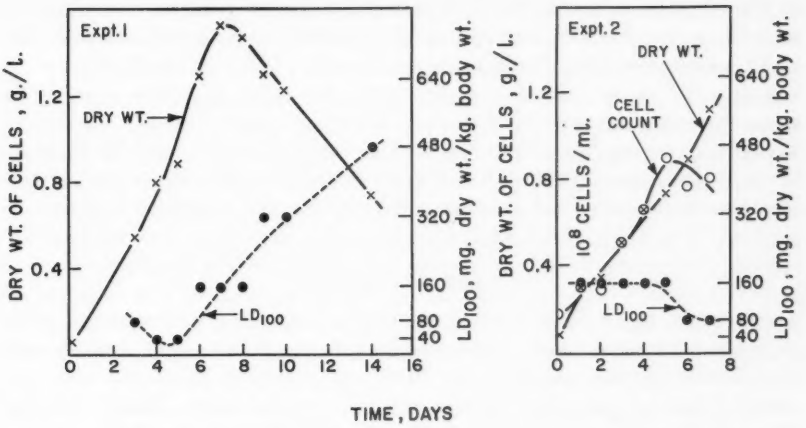

FIG. 1. The relation between production and accumulation of fast-death factor and developmental stage of a culture. Culture conditions as in Table III except light intensity 600 ft-c. Two male, 30-g. mice/dosage level.

after the cells had attained maximum toxicity and foaming had become very pronounced. The culture was no longer viable on the 14th day. In the second experiment, growth was somewhat slower than in the first and the LD_{100} of the cells did not change during the first 5 days. It decreased to 80 mg./kg. on the sixth day, when the dry weight of cells had reached about 0.8 g./l., and remained at this level on the seventh or final day. The cell count increased steadily during the first 5 days. The minimum generation time was $2\frac{1}{4}$ days. On the sixth and seventh days the cell count was lower than on the fifth. Despite this indication of lysis, the LD_{100} of the cells decreased during this period. FDF was detected in the culture filtrate on and after the third day, indicating that leakage and/or lysis was occurring even in the early stages of growth. It is therefore understandable that the minimum LD_{100} of the cells was not the same in the two experiments. Despite the uncertain losses of FDF, the LD_{100} of the cells in both experiments either reached or seemed about to reach a minimum when cell concentration reached similar values.

Discussion

This work has shown that it is possible to isolate a culture of a blue-green alga from a bloom, grow it in quantity, and have it produce cells that can be at least as toxic as the most toxic bloom that has ever been reported. Depending on culture conditions and treatments, the cells of *M. aeruginosa* NRC-1 had an LD_{100} ranging from 40 (Fig. 1) to greater than 480 mg./kg. (Table I). Shelubsky (21) has reported a minimal lethal dose of 125 mg./kg. for white mice injected intraperitoneally with either of two *Microcystis* blooms. Other reports do not give sufficient data about the dry weights

of the algae injected to make direct comparisons possible. However, a toxic bloom has been encountered in this study that consisted predominantly of *M. aeruginosa* and produced fast deaths at an LD_{100} of 40 mg./kg.

Both slow deaths and fast deaths appear to have been encountered by others in studies of blooms of various sorts but nobody, except Olson (17), seems to have suggested that more than one substance might be involved in algal poisonings. The present work, indicating that there are at least two factors produced by a single species in culture, confirms Olson's suggestion. The work of Wheeler, Lackey, and Schott (25) with blooms containing predominantly *M. aeruginosa* also indicated the involvement of at least two factors. They found that fresh material injected into mice caused deaths in 16 to 36 hours. After freezing and thawing or lyophilizing, the algae were 10 times more toxic, produced deaths in 40 to 90 minutes with symptoms like those produced by FDF.

The filtrates from some bloom collections have been reported as toxic (3, 6, 19, 22), non-toxic (15, 22), or toxic only after freezing (25) or decay (13). The involvement of more than one factor, especially one which is an endotoxin, whose detection depends not only on whether it is being produced but also on whether it is being liberated, helps to explain some of these conflicting observations. The release of an endotoxin might also explain the reported need for algal decomposition to promote the development of toxicity in certain bloom collections (10).

Olson (17) was the first to isolate and culture a strain of *M. aeruginosa* for toxicity studies. He reported parenteral lethal doses with mice ranging from 650 to 3400 mg. dry wt./kg. body weight but gave no survival times or symptoms. He concluded that it was not practical to use laboratory cultures for the study of toxicity since the extremely large quantities of algae that would be needed could most economically be obtained from lakes where winds had concentrated the material in a natural way. By a judicious choice of culture conditions and time of harvest, however, it now appears feasible to abandon the collection of blooms and instead to grow sufficiently large amounts of a strain like *M. aeruginosa* NRC-1 for the isolation and identification of the toxic factors. The difference in toxicity between Olson's strain and *M. aeruginosa* NRC-1 may represent a true strain difference. It may also reflect differences in culture conditions and/or the failure to release appreciable quantities of an endotoxin that may have been present.

Recently, Thompson, Laing, and Grant (24) have reported that lyophilized cells from a contaminated culture of another strain, *M. aeruginosa* (Western strain), grown on solution No. 11 were non-toxic when injected into mice at 480 mg./kg. A pure subculture of the same strain grown under a variety of culture conditions was similarly non-toxic. Bacteria isolated from the contaminated culture, however, produced slow deaths (3 to 48 hours) accompanied by symptoms of partial paralysis of the hind legs, difficulty in breathing, and death with convulsive twitching of legs. Lyophilized cells of a grossly contaminated culture (made by adding 1% pepticase to solution No. 11)

injected at 480 mg./kg. caused 70% mortality. This was increased to 100% after 18 hours incubation. These results suggest that bacterial contaminants are not too important as sources of toxin in normally growing cultures of *Microcystis*, unless, perhaps, as sources of slow-death factors. This might account for the differences in the amounts of SDF observed with *M. aeruginosa* NRC-1 in different experiments in which the culture conditions and time of harvest differed (Tables I, II, III). Much more work needs to be done, however, before the origins and nature of SDF and FDF are fully understood.

Acknowledgments

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CHARACTERISTICS OF AN EXTRACELLULAR PROTEINASE FROM MICROCOCCUS FREUDENREICHII[1]

I. HUSAIN[2] AND I. J. MCDONALD

Abstract

An extracellular proteinase from *Micrococcus freudenreichii* is described. The enzyme hydrolyzes casein and crystalline β-lactoglobulin rapidly and crystalline bovine plasma albumin very slowly. The enzyme is most active at 50° C. but is essentially inactivated in 15 minutes at 60° C. The optimum pH for enzyme activity is 5.5 to 6.4. The possible role of the proteinase in cheese ripening is discussed.

Introduction

One of the characteristic changes in the process of cheese ripening is the gradual increase in soluble nitrogen as a result of the hydrolysis of milk proteins by natural milk proteinases, rennet, and microbial proteinases. In recent years, several investigators (3, 4, 9) have studied proteinases of bacterial species isolated from Cheddar cheese. These authors have been concerned with intracellular enzymes and little has been done with enzymes that appear in the culture fluid. Some species of the genus *Micrococcus* produce easily detectable amounts of extracellular proteinase and may have a role in cheese ripening. Since *Micrococcus freudenreichii* has been found to improve Cheddar cheese flavor (1), it was chosen as a source of proteolytic enzyme and some of the characteristics of a crude preparation of its extracellular proteinases are reported.

Materials and Methods

Cultures of *M. freudenreichii* (A.T.C.C. No. 407) were maintained by monthly transfer on tryptone extract agar slopes (Bacto tryptone 0.5%, Bacto yeast extract 0.5%, and agar 1.5%). For enzyme production, 250 ml. of tryptone extract broth was inoculated with 10 ml. of a 24-hour broth culture of the organism and incubated at 30° C. for 4 to 5 days on a rotary shaker (A. H. Northcott, Guelph). The cells were removed by centrifugation and the clear supernatant frozen. The frozen medium was allowed to thaw slowly until one-half the original volume of liquid was collected. This concentrated culture fluid, which contained about 75% of the original enzyme activity, was saturated with ammonium sulphate at 0° to 4° C. and stirred for 2 hours. The precipitate was collected by centrifugation at 0° to −5° C. for 20 minutes at 20,000 × *g*. The precipitate was dissolved in 1/20th the original volume of water and dialyzed in a Visking casing for 5 hours against running tap water,

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[2]N.R.C. Postdoctorate Fellow, 1955–1956.

overnight against distilled water at 4° C., and for a further 2 hours against running tap water. The dialyzed preparations were frozen and could be stored at −10° C. for at least 3 months without loss of activity.

Casein, the usual substrate, was prepared by suspending 6.0 g. of vitamin-free casein (Nutritional Biochemicals Corp.) in 100 ml. of 0.05 *N* sodium hydroxide and boiling the suspension for 10 minutes. The solution was cooled, 7.5 ml. of 0.1% aqueous merthiolate (Lilly) added, the reaction adjusted to pH 6.3, and the volume made to 300 ml. Two per cent solutions of α- and β-casein were prepared in the same manner. Bovine plasma albumin (Armour) and β-lactoglobulin (Pentex, Inc.) were dissolved in 0.9% saline to give 2% solutions at pH 6.3. Merthiolate was added as above. The buffer (pH 6.3) used throughout, unless otherwise stated, contained 68 g. of sodium acetate trihydrate and 31 g. of boric acid per liter.

The reaction mixture for the assay of enzyme activity consisted of 5.0 ml. of substrate, 2.0 ml. of buffer, 2.0 ml. of water, 0.1 ml. of toluene, and 1.0 ml. of enzyme and, unless otherwise stated, was incubated at 35° C. Aliquots (3.0 ml.), removed at zero time and after 2 hours, were mixed thoroughly with 3.0 ml. of 0.6 *N* trichloroacetic acid to stop the reaction and filtered. Tyrosine was determined in aliquots of the filtrate using the method of Anson (2). Proteinase activity is expressed as the amount of tyrosine liberated from 1% protein solutions by 1 ml. of enzyme.

Results

Hydrolysis of casein by concentrated enzyme preparations from *M. freudenreichii* culture media was essentially proportional to the amount of enzyme added to the reaction mixture (Fig. 1). Substrate concentration also affected

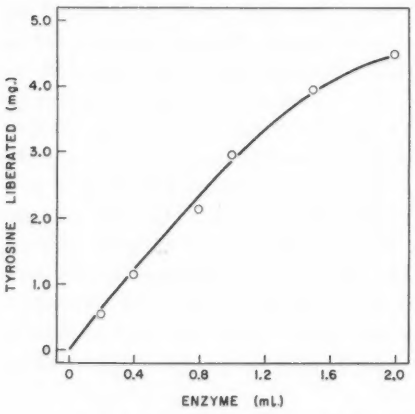

FIG. 1. Effect of concentration of micrococcus proteinase on liberation of tyrosine from casein. (Reaction mixture, 10 ml., incubated at 35° C. for 2 hours.)

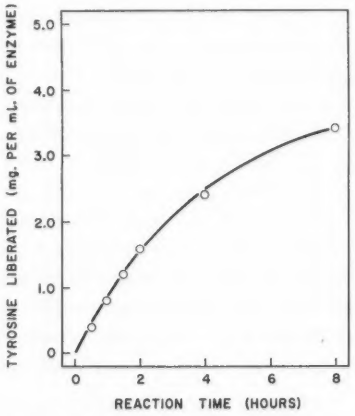

FIG. 2. Effect of reaction time on liberation of tyrosine from casein by micrococcus proteinase. (Reaction mixture, 10 ml., incubated at 35° C.)

the rate of proteolysis. The reaction rate rose rapidly as the casein concentration was increased from 0.2 to 0.4 mg. per ml. and more slowly as the casein concentration was increased from 0.4 to 0.8 mg. per ml. At higher concentrations of casein, the reaction rate leveled off or dropped slightly. A similar effect has been observed with proteinase from *Bacterium linens* (5).

Proteolysis of casein by micrococcus enzyme occurred essentially at a linear rate during the first 2 hours of reaction at 35° C., then decreased slowly (Fig. 2). This decrease may be the result of inactivation or inhibition of the enzyme or a combination of both.

The proteinase was most active at 50° C. (Table I). The activity dropped sharply above 55° C. and gradually below 45° C. At 35° C., activity was about one-half maximal but since this was a convenient temperature, it was used for routine assay of enzyme activity.

TABLE I

EFFECT OF TEMPERATURE ON RATE OF HYDROLYSIS OF CASEIN BY *M. freudenreichii* PROTEINASE

Temperature, °C.	Tyrosine, μg.*
10	30
20	100
30	300
37	500
40	560
45	900
50	1020
55	960
60	200

*Liberated by 1 ml. of enzyme in 2 hours.

TABLE II

INACTIVATION OF *M. freudenreichii* PROTEINASE BY HEAT

Temperature, °C.	Time, min.	Tyrosine, μg.*
50	0	1068
50	15	800
50	30	692
50	45	532
50	60	108
60	0	1040
60	15	80
60	30	80
60	45	20
60	60	80
100	0	1040
100	15	20
100	30	20

*Liberated by 1 ml. of enzyme in 4 hours at 35° C.

To determine the rate at which proteinase was inactivated at high temperatures, enzyme preparations were diluted with an equal volume of acetate–borate buffer at pH 7.0 (preliminary experiments having shown that the enzyme was most stable at this pH) and heated at the desired temperatures. Samples were removed at intervals, cooled immediately to 10° C., and assayed for activity on casein. The results (Table II) showed that heating at 50° C. for 30 minutes reduced proteinase activity by almost 50% and heating for 60 minutes essentially inactivated the enzyme. At 60° C. and 100° C., the enzyme was inactivated by heating for 15 minutes.

Hydrolysis of casein by *M. freudenreichii* proteinase was measured at different hydrogen ion concentrations. The most rapid hydrolysis occurred between pH 5.7 and 6.0 at 35° C. (Fig. 3). When the incubation temperature was 20° C., hydrolysis was most rapid at pH 6.4. A similar series of values obtained at 45° C. gave almost the same curve as presented for 35° C., but with a sharper peak at pH 5.7. The reaction for optimum activity, therefore, tended to become more alkaline as the incubation temperature was reduced. These results were obtained using acetate–borate buffer but experiments at 35° C. with other buffers indicated that the results were not influenced by the

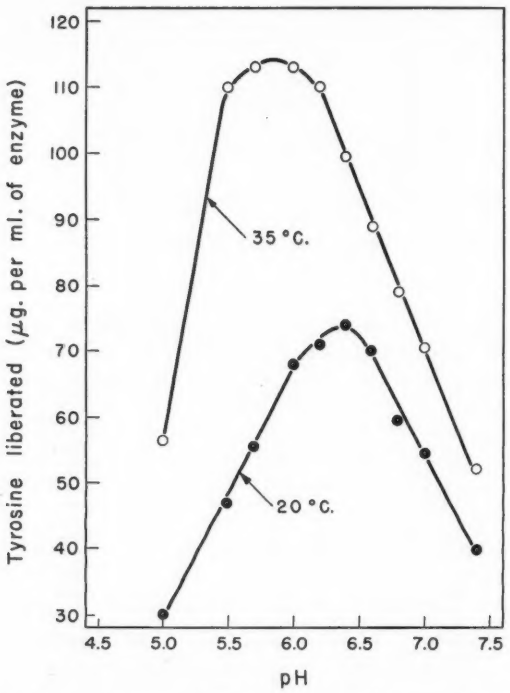

FIG. 3. Effect of temperature and pH on activity of micrococcus proteinase. (Reaction mixture, 10 ml., incubated 2 hours at 35° C. and 4 hours at 20° C.)

buffer used. Thus the optimum activity of the micrococcus proteinase occurred in the range pH 5.5 to 6.4. However, since early experiments indicated that the optimum pH for the enzyme was pH 6.3, this pH rather than pH 5.7 to 5.9 was used for routine enzyme assay.

Divalent metal ions did not appear to affect the proteolytic activity of enzyme preparations. Activity was not increased by the presence of 0.005 *M* concentrations of calcium chloride, ferric chloride, ferrous sulphate, magnesium sulphate, manganous sulphate, zinc chloride, or 0.0005 *M* concentrations of cobaltous chloride or cupric sulphate, and was not reduced by the presence of 0.2 *M* ethylenediamine tetraacetic acid in the reaction mixture.

Enzyme preparations hydrolyzed proteins other than casein. Of proteins tested, α-casein was hydrolyzed most rapidly followed in order by casein, β-lactoglobulin, β-casein, and bovine plasma albumin (Table III). Plasma albumin that had been boiled for 10 minutes was hydrolyzed as readily as β-casein but β-lactoglobulin that had been boiled was not altered as a substrate for the enzyme. In other experiments, denatured hemoglobin was hydrolyzed. Reaction of the enzyme with casein or β-casein caused the mixture to become milky or opalescent as has been reported for a proteolytic enzyme from *Bacterium linens* (5). Rennin, on the other hand, made casein and α-casein opalescent but did not affect β-casein.

TABLE III

HYDROLYSIS OF DIFFERENT PROTEINS BY *M. freudenreichii* PROTEINASE

Substrate*	Tyrosine, mg.†
Casein	1.94
α-Casein	3.49
β-Casein	1.12
β-Lactoglobulin	1.55
Bovine plasma albumin	0.26

*Substrate concentration, 1.0%.
†Liberated by 1 ml. of enzyme in 2 hours at 35° C.

Discussion

The role of bacterial proteinases in the process of cheese ripening has been discussed by several authors (3, 4, 9). It is generally agreed that results obtained with isolated purified bacterial enzymes cannot be directly applied to cheese ripening. However, available data indicate that the proteolytic enzymes produced by several bacterial species may be involved in the ripening process. Alford and Frazier (1) showed that the addition of certain strains of *M. freudenreichii* during the manufacturing process improved the flavor of Cheddar cheese and the results presented here indicate that *M. freudenreichii* is capable of hydrolyzing milk protein. Several bacterial proteinases have been reported (3, 4, 5, 6, 7, 8, 9) but of those associated with the cheese-ripening process, the enzyme from this strain of *M. freudenreichii* is one of the few that

is optimally active at an acid pH. However, the shift in pH optimum towards the alkaline range at low temperatures suggests that the enzyme may be only slightly active in cheese ripening but it is difficult to assess its actual importance since the micrococcus proteinase has not been studied at 7° to 15° C., the temperatures at which cheese is cured.

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THE INFLUENCE OF OXYGEN ON THE REDUCTION OF NITRITE BY WASHED SUSPENSIONS OF ADAPTED CELLS OF ACHROMOBACTER LIQUEFACIENS[1]

V. B. D. Skerman, Barbara J. Carey, and I. C. MacRae

Abstract

The influence of oxygen on the reduction of nitrate by adapted cells has been studied using a strain of *Achromobacter liquefaciens* which reduces nitrite but which does not reduce nitrate. The results have shown that active nitrite reduction occurs in the presence of oxygen and that the magnitude of the reduction is independent of the oxygen concentration in solution when the latter is a positive value. As the oxygen demand exceeds the supply however, the rate of nitrite reduction increases as the supply decreases.

Nitrite exerts an inhibitory effect on the reduction of oxygen which, at low concentration, appears to be due purely to competition for electrons. At higher concentrations the inhibition appears to be directed towards the oxygen-reducing enzymes.

Weissenberg (6) reported that the reduction of nitrite is especially susceptible to the inhibitory action of oxygen. Sacks and Barker (2) claimed that 2.5% oxygen in the gas phase inhibited the reduction of nitrite by 45% as compared with the anaerobic control and that air inhibited reduction by 65 to 75%. The degree of inhibition of nitrite reduction considerably exceeded that of nitrate reduction. Kefauver and Allison (1) have also claimed that considerable reduction of nitrite occurs in the presence of air.

The relationship between oxygen concentration and nitrite reduction has been examined in this laboratory using an organism which has the characters of *Achromobacter liquefaciens* (Eisenberg) Bergey *et al.* as outlined in the seventh edition of Bergey's Manual of Determinative Bacteriology. This culture (strain 39) was isolated from Victorian soil by Dr. Nancy Millis and is the same strain as that used by Dr. Jean Youatt (7). It does not reduce nitrate but reduces nitrite quite rapidly. This latter character does not appear in the original description of *A. liquefaciens*.

Materials and Methods

The preparation of washed suspensions and the polarographic equipment used in studying the action of oxygen on the function of nitrite-reducing enzymes are identical with those used in a previous publication (Skerman and MacRae (4)). The only significant variation was in the conditions of growth of cultures, the analysis of nitrite, and the time of addition of nitrite in the experiments. Ordinates in all polarograms are identical with those quoted in the previous paper.

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Contribution from the Department of Bacteriology, University of Queensland, Brisbane, Australia.

Analysis of Nitrite

Polarographic procedures so far examined have proved unsatisfactory for nitrite analysis. The method of Rider and Mellon used in a previous study by Skerman, Lack, and Millis (5) was employed. Nitrite estimations were made from standard curves prepared with pure nitrite solutions.

No significant difference was found between the color densities obtained with pure solutions of nitrite and with solutions in which the final concentrations of buffer, substrate, and bacterial cells were included.

The reduction of nitrite by adapted cells proceeds rapidly even in the presence of oxygen. In order to arrest this reaction to permit analysis of residual nitrite, formalin and phenol were tested. Neither interferes with the colorimetric analysis but inactivation by formalin was too slow and phenol formed an emulsion in the phosphate buffer and had to be discarded. Simple acidification to pH 2.0 with hydrochloric acid was effective. Its use necessitated a study of its effect on the reduction of nitrite, both alone and in the presence of the substrates—particularly the amino acids—used in the experiments.

Skerman and MacRae (3) showed that the rate of reduction of nitrite in the presence of urea in acid solutions was dependent on the concentration of the reacting substances. In the present investigations two concentrations of acid were tested. In the *dilute* series 5 ml. of the *M*/40 phosphate buffer containing the substrate and nitrite were added to 35 ml. 0.037 *N* HCl and diluted with the acid to 50 ml. This concentration of acid gave a final pH of 2.0. In the *concentrated* series 5 ml. of the *M*/40 phosphate buffer mixture was added to 5 ml. 0.26 *N* HCl giving a final pH of 2.0. The mixture was allowed to react in this state and diluted to 50 ml. just prior to sampling for analysis. All samples were further diluted 1/50 during the course of analysis.

Fig. 1 shows results obtained with urea, *dl*-alanine, and yeast extract in which analyses were made initially and at the 30-minute interval only. The rate of reduction in dilute acid with yeast extract and *dl*-alanine does not differ significantly from that in the dilute acid alone. The initial nitrite concentration was limited to the 200 p.p.m. used in most of the subsequent work.

Variation of the concentration of *dl*-alanine from 1.5 to 10 mg. per ml. in the original buffer mixture did not cause any significant increase in the rate of nitrite reduction in the dilute acid. The latter increases with increasing concentrations of nitrite, but over the range of 50 to 200 p.p.m. nitrite the presence of 1.5 mg. *dl*-alanine per ml. in the original buffer mixture did not accelerate the reaction.

These experiments showed that the rate of nitrite reduction was very slow when the sample was rapidly diluted in hydrochloric acid and that *dl*-alanine did not influence the rate to any significant extent. Since the primary dilution was immediately followed by a further dilution of one 1/50 and by reaction of the nitrite with the sulphanilic acid the reduction of nitrite occurring during the period of sampling could be ignored.

Growth of Achromobacter and Preparation of Cell Suspensions

The yeast extract – peptone medium employed in previous studies was again used as the basal medium with a final pH of 7.2. In a medium containing 1300 p.p.m. nitrite ion, rapid growth occurred with complete reduction of the nitrite in 20 to 24 hours. This was accompanied by vigorous gas production. The change in density of the culture was followed at 24-hour intervals in the same basal medium with various concentrations of nitrite. The results are shown in Fig. 2. It is significant that initial development was more rapid in the basal medium plus 1000 p.p.m. nitrite than in the basal medium alone. With increasing concentrations of nitrite the initial rate of growth was delayed although the subsequent rate was rapid. Nitrite had completely disappeared from the cultures containing 1000 p.p.m. nitrite ion in 24 hours and from the 2000 p.p.m. before 48 hours. Traces were still present in the 3000, 4000, and 5000 p.p.m. at 72 hours.

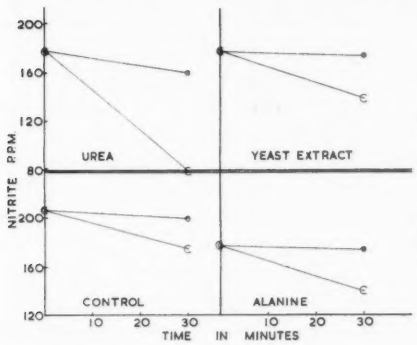

FIG. 1. The influence of urea, yeast extract, and *dl*-alanine in concentrations of 0.15 mg. per ml. on the reduction of nitrite in *dilute* (O—O) and *concentrated* (C—C) acid solutions (see text).

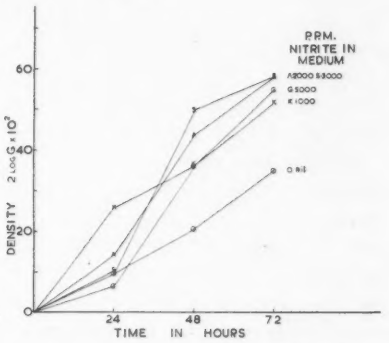

FIG. 2. Growth of *A. liquefaciens in* yeast extract – peptone water containing varying concentrations of nitrite.

Equal densities of cells harvested from the 1300 p.p.m. and 4000 p.p.m. cultures at a stage when nitrite had just disappeared had approximately the same nitrite-reducing power. For this reason the lower concentration which yielded suitable cells in 24 hours was used for all subsequent work.

Cells harvested at 24 hours and suspended in $M/40$ phosphate buffer at pH 7.2 at a density of 5×10^9 cells per ml. retained their activity for a period of at least 48 hours. Similar densities of cells were examined for change of activity after 24 hours at 28° C. following incubation anaerobically in the presence of (*a*) 0.1 M lactate, (*b*) 0.1 M lactate and 100 p.p.m. NO_2^-, (*c*) 0.1 M lactate and 0.1% $(NH_4)_2SO_4$. An $M/40$ phosphate buffer was used in all cases and separately as a control. After incubation, cells were recentrifuged, washed, and resuspended. No significant change occurred except with cells incubated in 0.1 M lactate in which there was a reduction in activity of about 60%. Cells grown in the yeast extract – peptone water containing

nitrite were not subject to further adaptation in the manner reported by Youatt (7) for cells grown on agar plates. When held overnight as packed suspensions in centrifuge tubes activity was markedly diminished.

Cells of *Achromobacter* stored overnight in *M*/40 phosphate buffer at a density of 5×10^9 cells per ml. at 4° C. were used throughout the study. The cells were centrifuged and resuspended to the required density before use.

Substrates

The object of our experiments was to study the reduction of nitrite in the presence of various concentrations of dissolved oxygen. The primary aim in selecting a substrate was to obtain one upon which the organism could actively respire at a uniform rate over the period of the experiment. Difficulties similar to those reported in the previous paper were encountered.

When the oxygen-uptake rate of cells grown on yeast extract–peptone agar was measured on lactate, formate, succinate, acetate, fumarate, and citrate substrates, lactate was the only carbon substrate on which there was any pronounced activity. Citrate completely inhibited endogenous respiration. Growth of the organism on the yeast extract–peptone agar in the presence of lactate did not improve the lactic dehydrogenase activity.

Lactate in a final concentration of 0.1 *M* was tested over a period of 30 minutes with a cell density of 5×10^8 cells per ml. and a gassing rate of 100 ml. air per minute through 50 ml. of solution. The final oxygen-uptake rate (B) was significantly less than the initial uptake rate (A) (Fig. 3). The gradual change in the oxygen "steady state" (C) throughout the 30-minute period suggested that the change was not due to lack of substrate (see Skerman and MacRae (4)). In addition, the maximum concentration of lactate oxidizable during the 30-minute period, based on the maximum initial oxygen-uptake rate and on the first stage oxidation of lactate to pyruvate, was only 0.005 *M*.

When the oxygen-uptake rate was examined with various concentrations of lactate there was an *apparent* reduction with increasing molarities of lactate as shown in Fig. 4. This was found to be due, in all cases except the 0.4 *M* lactate, to an increase in the magnitude of the residual current and an alteration in the oxygen concentration: diffusion-current ratio with increasing concentrations of lactate. That such an alteration occurs with change in substrate concentrations has already been emphasized in the analysis for nitrate (Skerman and MacRae (3)) and should always be borne in mind where polarographic methods are used in biological studies. When compensation was made for the change in the $(O_2)/I_d$ ratio the slopes for 0.025 *M*, 0.05 *M*, 0.1 *M*, and 0.2 *M* lactate were identical. Some suppression of the oxygen-uptake rate was evident with 0.4 *M* lactate.

It is quite clear that the change in oxygen-uptake rate occurring during the 30-minute aeration period is not due to change in substrate concentration. Continuation of aeration for a further 30 minutes resulted in a further decrease in oxygen-uptake rate but to a less marked degree, the relative rates in one

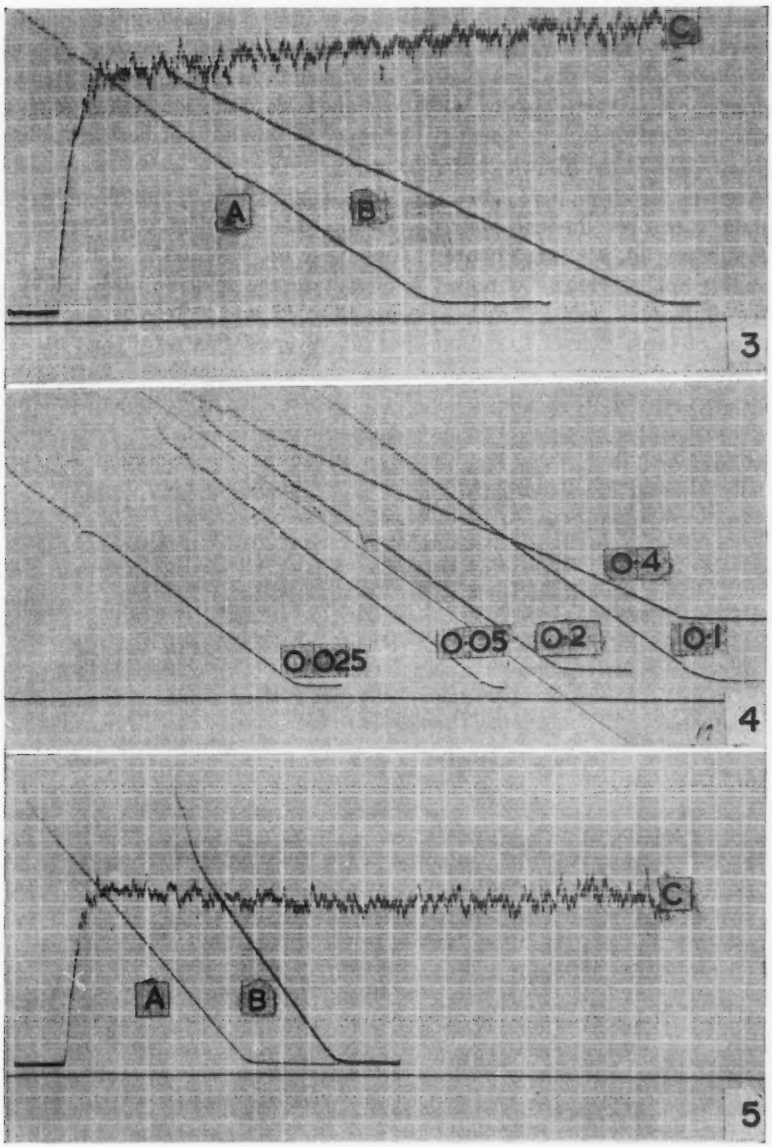

FIG. 3. Relative rates of oxygen reduction by *A. liquefaciens* on 0.1 *M* lactate. A, initial reduction rate; B, reduction rate after 30 minutes' aeration; C, "steady state" oxygen recording during 30 minutes' aeration.

FIG. 4. *Apparent* relative rates of oxygen reduction by *A. liquefaciens* on 0.025 *M*, 0.05 *M*, 0.1 *M*, 0.2 *M*, and 0.4 *M* lactate.

FIG. 5. Relative rates of oxygen reduction by *A. liquefaciens* using 3.0 mg. of yeast extract per ml. A, initial reduction rate; B, reduction rate after 30 minutes' aeration; C, "steady state" oxygen recording during 30 minutes' aeration.

experiment using 0.2 *M* lactate being 7.3, 5.1, and 4.5 at 0, 30, and 60 minutes, respectively. Prior aeration of the cells had no influence on their behavior towards oxygen in the presence of lactate and the same general trend in reduction in oxygen uptake with continued aeration was found with all concentrations of lactate tested. The cause of this has not been elucidated. Mixing 5% CO_2 with air had no effect.

A search for alternative substrates which might result in a uniform oxygen-uptake rate over the half-hour period was made. Difco yeast extract at 0.1 mg. per ml. was inadequate; 1.0 mg. per ml. resulted in a measure of stability not previously obtained with lactate the relative rates before or after 30 minutes' aeration with air being 48 to 45, and increasing the concentration of yeast extract to 3.0 mg. per ml. reversed this ratio (Fig. 5). An examination was therefore made of the amino acid oxidation of *A. liquefaciens*. Aspartic acid, glutamic acid, *dl*-alanine, arginine, threonine, lysine, and leucine were tested. The order of activity was *dl*-alanine (44), aspartic acid (33), glutamic acid (31), and arginine (18.5). Threonine, lysine, and leucine showed no activity.

Both glutamic and aspartic acid, when used at a concentration of 1 mg. per ml. showed the same general decrease in oxygen uptake over the 30-minute period as did lactate, the change being more pronounced with the aspartate. *dl*-Alanine, used at 1.5 mg. per ml., showed a slight increase in rate over the 30 minutes, the ratio being 1:1.1. Although strict uniformity in oxygen-uptake rate was not obtained with *dl*-alanine the change in the steady state oxygen concentrations was so slight that the use of the substrate was adopted.

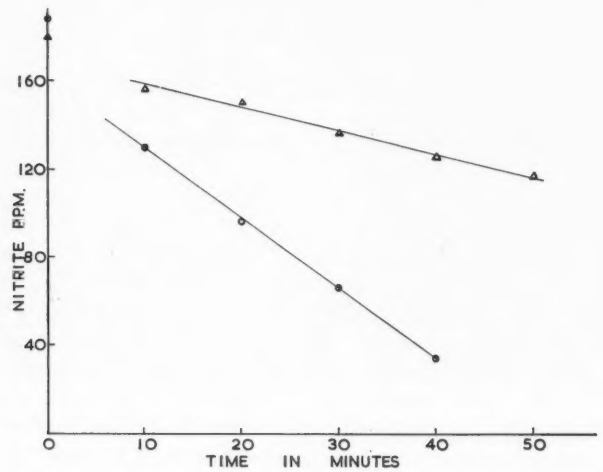

FIG. 6. Rate of reduction of nitrite by *A. liquefaciens* under anaerobic conditions with cells of two different ages.

Results

The Reduction of Nitrate under Anaerobic Conditions

Two curves showing the rate of reduction of nitrite by two suspensions of different ages of *A. liquefaciens* are shown in Fig. 6. These curves were plotted from data obtained by colorimetric determination of nitrite under anaerobic conditions. Similar results were obtained by measuring nitrogen evolution in the Warburg apparatus.

The Influence of Nitrite on the Reduction of Oxygen

Initial experiments were conducted in a manner identical with that reported for nitrate by Skerman and MacRae (3). It was immediately obvious that (i) the presence of nitrate reduced the rate of oxygen utilization and

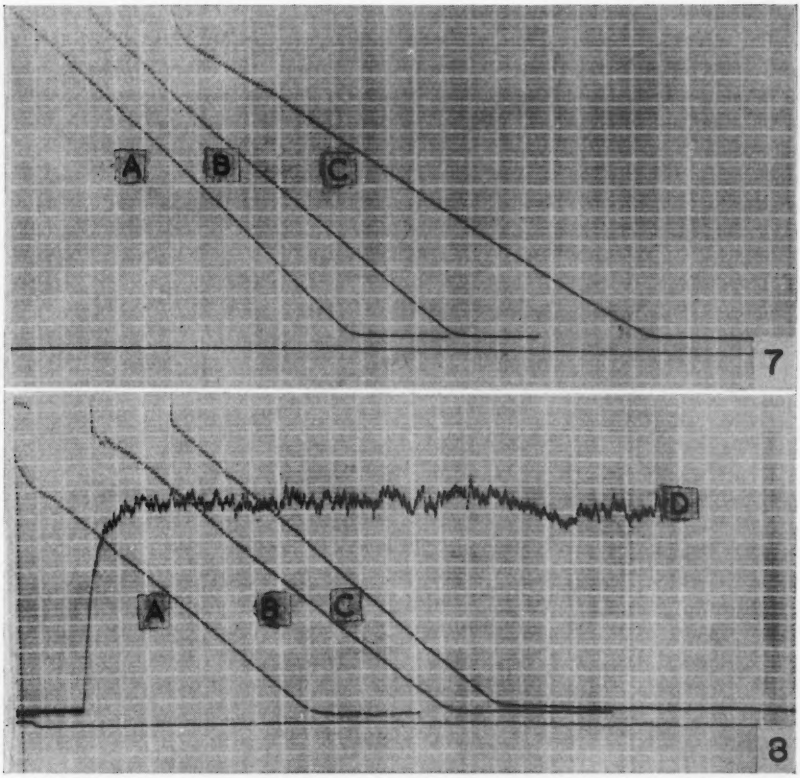

FIG. 7. The influence of nitrite on the reduction of oxygen by *A. liquefaciens*. A, reduction rate in the absence of nitrite; B, similar to A but diluted with one-ninth of its volume of water; C, similar to A but diluted with one-ninth of its volume of a solution containing 500 p.p.m. NO_2^-.

FIG. 8. Influence of 30 minutes' aeration on the availability of substrate to *A. liquefaciens*. A, initial rate of oxygen reduction using 1.5 mg. *dl*-alanine per ml.; B, rate of reduction immediately after dilution of A with one-ninth of its volume of water; C, similar to B but after 30 minutes' aeration with air; D, "steady state" oxygen recording during the 30 minutes' aeration.

(ii) nitrite was reduced rapidly even in the presence of oxygen. Before proceeding with the study of the latter, it was necessary to ascertain the significance of the former.

Fig. 7 shows the oxygen-uptake rate before (A) and after (C) the addition of nitrite. Curve B represents a similar experiment in which water was substituted for the nitrite. The reduction in oxygen-uptake rate in B is due to dilution only.

Fig. 8 shows the results of a similar experiment in which the initial oxygen-uptake rate (A) was plotted; 5 ml. of water added and reaerated and the oxygen-uptake rate plotted (B). The mixture was then aerated for 30 minutes and the uptake replotted (C). It is clear from the experiment that the concentration of substrate was adequate and not the cause of the change occurring on the addition of nitrite.

The effect of various final concentrations of nitrite (50, 100, 200, 400, 800, and 1600 p.p.m.) was then examined. In all cases the effect on the oxygen-uptake rate was immediate. The results of two of these experiments are shown in Fig. 9. The reduction in oxygen-uptake rate is practically uniform up to 200 p.p.m.; with higher concentrations there is a progressive reduction

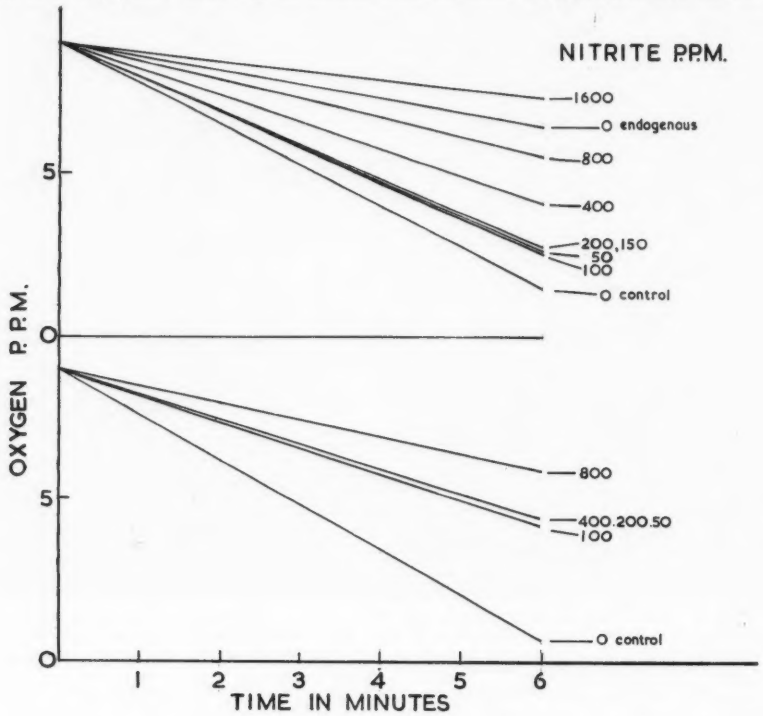

FIG. 9 The influence of nitrite concentration on the rate of reduction of oxygen by *A. liquefaciens*.

to and beyond the endogenous rate. The concentration at which the latter change occurs appears to vary with different suspensions and may be related to the nitrite-reducing capacity of the suspension.

Warburg and polarographic determinations have shown that the rate of nitrite reduction anaerobically over the range of 50 to 1600 p.p.m. nitrite is independent of the nitrite concentration.

Observations made on the oxygen-uptake rate following complete reduction of the nitrite using initial concentrations of 50, 100, and 150 p.p.m. nitrite have shown that the nitrite has no residual effect on the oxygen-reducing system over this range (Fig. 10).

No observations have been made of the immediate effect of concentrations of nitrite below 50 p.p.m. Such experiments are technically difficult to perform because the nitrite-reduction rate of the suspensions is of the order of 3 p.p.m. per minute and most experiments cannot be performed in less than 10 minutes.

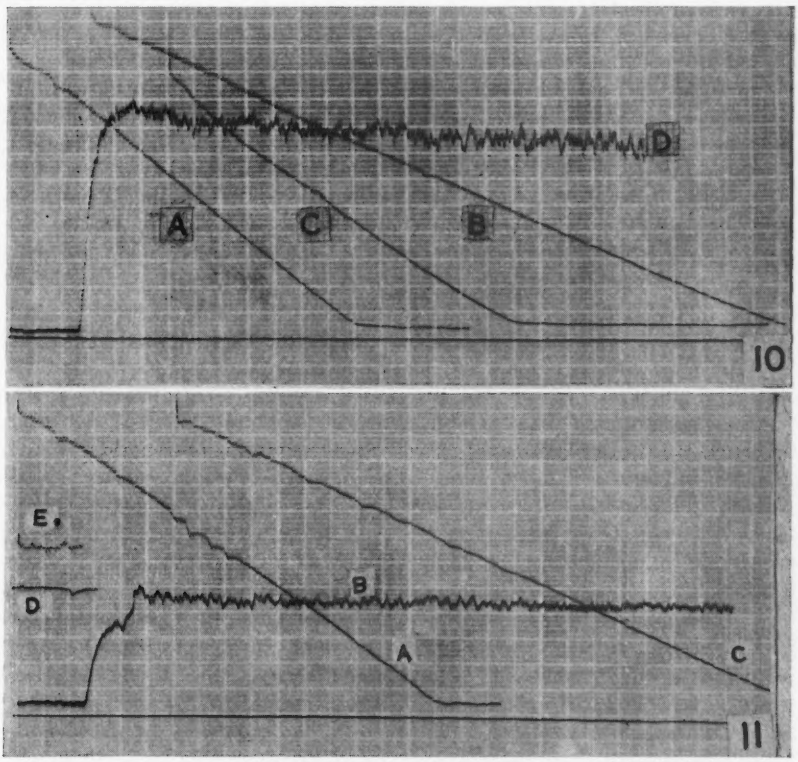

FIG. 10. Rate of reduction of oxygen by *A. liquefaciens*. A, initially before the addition of nitrite; B, immediately after the addition of one-ninth of the volume of a solution containing 500 p.p.m. NO_2^-; C, after 30 minutes' aeration with air following the addition of nitrite; D, "steady state" oxygen recording during the 30 minutes' aeration.

FIG. 11. For explanation see text.

Fig. 10 shows the results of an experiment in which an initial concentration of 50 p.p.m. nitrite was present in the cell. Curve A shows the oxygen-reduction rate immediately prior to the addition of the nitrite and curve B immediately afterwards. Curve C shows the reduction rate following 30 minutes' aeration with air. All the nitrite was reduced after the first 15 minutes of aeration. This reduction of nitrite is reflected in a gradual decrease in the oxygen "steady state" level which is consistent with the removal of the final traces of nitrite. The reduction of the nitrite begins as soon as the nitrite is added. During the time taken to plot curve B (approximately 10 minutes) 20 to 30 p.p.m. of nitrite could be removed. It is significant, however, that the oxygen-reduction rate during this period is very uniform indicating that the initial effect of nitrite is uniform to concentrations as low as 20 p.p.m. and possibly lower.

The Influence of the Oxygen Concentration on the Reduction of the Nitrite

In view of the fact that the concentration of nitrite employed influences the rate of oxygen uptake beyond certain limits, the concentration of nitrite selected for this study was chosen from the lower range over which the effect was uniform, and of such a magnitude that the residual nitrite after a 30-minute period was sufficient to ensure that the rate of oxygen reduction would not alter during the course of the experiment. For this reason the concentration of 200 p.p.m. was chosen for all subsequent experiments.

The following technique was adopted: 35 ml. *M*/40 phosphate buffer at pH 7.0: 5 ml. of cell suspension and 5 ml. of substrate were mixed in the cell and gassed with oxygen. The initial oxygen-uptake rate was then plotted (Fig. 11, A). The recording polarograph was then returned to zero and the mixture in the cell gassed with the gas mixture till saturation was reached (usually 2 minutes). Five milliliters of the nitrite solution was then added

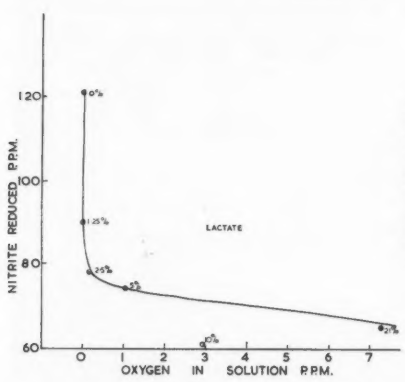

FIG. 12. The influence of the oxygen concentration in solution on the reduction of nitrite by *A. liquefaciens* using 0.1 *M* lactate as a substrate.

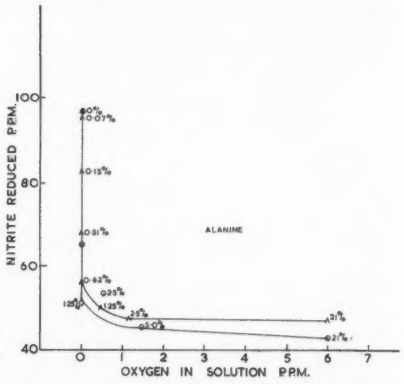

FIG. 13. The influence of the oxygen concentration in solution on the reduction of nitrite by *A. liquefaciens* using 1.5 mg. *dl*-alanine per ml. as a substrate (two series).

and samples removed for analysis after 1 minute and again after a further 30 minutes during which time the oxygen concentration was continually recorded (Fig. 11, B). The final oxygen-uptake rate was then recorded (Fig. 11, C). Curves D and E in Fig. 11 represent the "still" and "agitated" oxygen recordings for the gas mixture in the cell-free buffer (see Skerman and MacRae (4) for detailed explanation). By varying the gas mixture the reduction of nitrite could be determined in relation to the oxygen concentration computed from the "steady state" curves.

The results of three experiments are shown in Figs. 12 and 13.

Discussion

The observations on the methods of sampling and analysis of nitrite merit little further comment other than to emphasize the relative inactivity of amino acids and nitrite in dilute acid solutions and draw attention to the effect of this on analysis of amino acids in small concentrations by the nitrite method.

The marked loss of activity of cells held overnight in dense packed suspensions as compared with similar cells dispersed in a phosphate buffer merits further study.

The variation in the rate of respiration of *A. liquefaciens* on various substrates over short-term experiments under high degrees of aeration follows a pattern similar to that found with *Pseudomonas denitrificans*. This warrants further investigation because of its importance in respiration studies in general. The availability of substrates upon which this variation was negligible obviated any necessity to study the matter at this stage.

An influence of nitrite on the rate of oxygen reduction was not wholly unexpected in view of the biological toxicity of nitrites.

The relatively constant effect of nitrite (see Fig. 9) in the lower concentrations (50–200 p.p.m.) suggested that the nitrite was competing with the oxygen for the available donor electrons (*vide infra*). The progressive inhibition at higher concentrations of nitrite suggests interference of an entirely different nature which would appear to be directed towards the oxygen-reducing enzymes since the rate of nitrite reduction anaerobically on the same substrate is independent of the concentration of nitrite over the same range. This aspect is being further investigated. It suffices to emphasize at this stage that great variation in results obtained in a study of this nature must be expected if the influence of the nitrite concentration is ignored.

This inhibitory action of nitrite on oxygen reduction must also affect the growth of cells in media containing nitrite. The density curves shown in Fig. 2 were obtained in vessels exposed to air. Even with the lowest concentration of nitrite employed inhibition would have been considerable. The ready availability of the nitrite in solution, coupled with the low solubility and slow diffusion rate of oxygen, probably explains the greater suitability of the medium plus 1000 p.p.m. nitrite compared with the medium alone over the first 24 hours and for the greater suitability of all nitrite containing

media over the 72-hour period. The reduced rate of initial development in media with higher nitrite concentrations may have been due to an almost total inhibition of oxygen reduction coupled with difficulty experienced by the organisms in adapting to nitrite in the presence of the oxygen. This has yet to be investigated.

No residual effect of the nitrite on the oxygen-reduction rate has been detected in experiments in which complete reduction has taken place (Fig. 10). With a concentration of 200 p.p.m. in which the effect of nitrite has been determined initially and after 30 minutes, the absence of any further change in the rate of oxygen reduction during the 30-minute period indicated that this higher concentration of nitrite had no injurious effect on the oxygen-reducing enzymes and that the continued exposure to oxygen had no effect on the nitrite-reducing enzymes since a change in either would have been reflected in a change in the oxygen-reduction rate.

Parallel experiments conducted with *P. denitrificans* have yielded different results. These will be reported in a subsequent paper.

In studying the influence of oxygen on the reduction of nitrite a level of 200 p.p.m. nitrite was selected for reasons already indicated. The results shown in Figs. 12 and 13 indicate clearly that nitrite is reduced in the presence of oxygen. This finding is in agreement with that of Sacks and Barker (2) and Kefauver and Allison (1). In the figures the oxygen concentration in the gas phase is indicated in percentages for comparison with the concentration in solution. Because of the reduced rate of oxygen reduction in the presence of nitrite adequate oxygenation of the medium was accomplished with much lower concentrations of oxygen than that required for *P. denitrificans*. The amount of nitrite reduced diminishes progressively as the oxygen concentration in the gas phase is increased. When the latter reaches levels beyond which the oxygen first becomes detectable in the solution, the rate of nitrite reduction remains relatively constant with increasing oxygen concentration in the solution up to near equilibrium with air. This fact, coupled with the fact that over the lower concentrations of nitrite the effect of nitrite on the rate of oxygen reduction is also constant, suggests that the nitrite is competing with the oxygen for the available donor electrons and is not, at this concentration, exerting any other inhibitory effect.

The constancy in the degree of nitrite reduction over varying positive oxygen concentrations in solution can be explained in terms of the rate of oxygen reduction by the cells. As with *P. denitrificans* the oxygen reduction by *Achromobacter* is a zero-order reaction at all concentrations above a very low critical value below which the rate is a function of the oxygen concentration and the reaction is of the first order. As the concentration of oxygen in solution diminishes below this critical level the rate of nitrite reduction will increase to a maximum. The conditions existing in the solution in this stage would be similar to that discussed in the previous paper by Skerman and MacRae (4) in which it was argued that only a portion of the cell population was receiving any oxygen. Once the critical oxygen level is reached

the rate of reduction of oxygen becomes constant and consequently its effect on the reduction of nitrite becomes constant provided that the oxygen concentration per se is not injurious to the nitrite-reducing enzymes. There was no evidence to suggest the latter in any of the experiments.

Some calculations which have been made of the total number of electrons involved in the reduction of the oxygen and nitrite as compared with that involved in reduction of oxygen in the absence of nitrite suggest that the rate of oxidation of the substrate is limited by the rate of oxygen reduction. This aspect is receiving further attention.

A quite different relationship may exist as the nitrite concentration is increased to levels which exert a complete inhibitory effect on the oxygen-reducing enzymes. This aspect is also receiving further attention.

Although it is premature to draw comparisons with this work and that of Sacks and Barker, who used a culture of *P. denitrificans*, the publication of Kefauver and Allison (1) merits some comment. Their results on the growth of *Bacterium denitrificans* Chester (Figs. 2–6) indicate quite clearly that with increasing oxygen concentrations in the gas phase there is a progressive delay in the reduction of nitrite with its eventual cessation when the gas phase contained 20% oxygen. There is no evidence to suggest that nitrite is being reduced in the presence of detectable oxygen in solution. The evidence presented closely parallels that published by Skerman, Lack, and Millis (5) for *P. denitrificans*. Skerman and MacRae (3) indicated that such experiments do not discriminate between the effect of oxygen on the formation of, and the effect of oxygen on, the function of the nitrate- (or nitrite-) reducing enzymes and further (4) that it is more likely that only a portion of the population is getting the oxygen and the remainder is reducing nitrate (or nitrite).

The authors do not question the possibility of free oxygen being present in the solution in Fig. 6 (Kefauver and Allison) although the only evidence presented is the indirect evidence of no nitrite reduction which is in direct conflict with their observations on resting cells. Their results could be, and possibly are, due to the failure of the cells to form the nitrite-reducing enzymes in the presence of an adequate oxygen supply. This interpretation would explain the results obtained in Figs. 2–5 where it would appear that the delay in the reduction of nitrite corresponds to the period taken for the cells to increase in density sufficiently to reduce the oxygen concentration detectable in the solution to 'zero'* and create a condition in which adaptation to nitrite could occur. This would still be a valid interpretation even if the cells used as the inoculum were preadapted (see Skerman *et al.* (5)).

Kefauver and Allison do not state the nature of the substrate employed in the study of nitrite reduction with "resting", or non-proliferating cells. Their claim that such cell populations reduce nitrite in the presence of oxygen is in full accord with the authors' observations but it must again be emphasized that they have produced no evidence to sustain their claim of "excellent

*The authors' use of the expression 'zero' (5) was not meant to emphasize the state but to advise caution in its interpretation.

aeration". Similar results could be obtained with an aeration procedure which failed to maintain a positive oxygen level in the solution. The same criticism could also be applied to their Warburg experiment.

The demonstration of simultaneous metabolism of oxygen and nitrite by a population of cells without a direct estimation of the available oxygen in solution does not constitute proof that individual cells can metabolize nitrite in an environment containing free oxygen.

The procedure adopted in attempting to correlate Eh measurements with nitrite reduction using the poising method employed by Kefauver and Allison is subject to serious errors. The medium employed is too complex to permit any critical interpretation of data obtained; there is no guarantee that the electrode is not registering the redox potential of a system or systems involving hydrogen ions for which pH data would also be required and there is no guarantee that the bright platinum electrode is not functioning, in part at least, as an oxygen electrode. Furthermore, it is subject to poisoning during the long period of immersion in the complex organic solution.

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OBSERVATIONS ON FUNGI ASSOCIATED WITH PLANT ROOTS[1]

E. A. PETERSON

Abstract

Plant age and soil type influence the nature of the fungal flora associated with plant roots. Total numbers of fungi in the rhizosphere of wheat, as determined by the dilution technique, increased with plant age but the degree of fungal stimulation appeared to depend upon soil type. Penicillia and certain species of the Mucorales were relatively more abundant on the root surface and in the rhizosphere at the seedling stage than at later stages of growth. Species of *Fusarium* and/or *Cylindrocarpon* (depending upon soil type) and various sterile dark fungi were predominant on the roots of healthy red clover and wheat, but were relatively rare in rhizosphere and non-rhizosphere soil.

Introduction

The roots of plants growing in soil provide a unique habitat, the rhizosphere, which is particularly favorable for the development of soil microorganisms. Symbiotic relationships, such as occur between most legumes and nodule-forming bacteria or in mycorhizal formations, also are initiated in the rhizosphere. In contrast, certain undesirable organisms may become active in the vicinity of the roots and prove injurious to the plant by parasitic attack. However, the majority of microorganisms stimulated by plant roots are usually harmless saprophytes which live mainly on root excretions and on dead, decomposing root tissues.

For many years most of the published work on the microbiology of the rhizosphere dealt primarily with quantitative aspects. More recently, however, the trend has been to study the qualitative as well as the quantitative effects of plants on the soil microflora. Although emphasis has been placed largely on bacteria, other groups, particularly the soil fungi, have received some attention. Soils differing in reaction have been found to affect the predominance of certain fungi on corn roots (11). Qualitative as well as quantitative differences between fungi of rhizosphere and non-rhizosphere soil have been reported (6, 12). Information, pertaining largely to fungi which are in an active mycelial state, has been obtained by examination of the fungal populations of thoroughly washed roots of wheat (10), beech seedlings (3), and rye grass (12). Light intensity has been found to exert a marked effect on the composition of the fungal flora of beech-seedling roots (4).

Comparatively little is yet known about the nature of the fungal population associated with the roots of healthy plants. The present studies were conducted in an attempt to obtain further information on this subject. Emphasis has been placed on the influence of plant age and soil type.

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Contribution No. 452 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa, and the Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Herts., England.

Methods

Atle wheat and late flowering Montgomeryshire red clover were grown in 4-in. glass pots filled with natural soil which had been thoroughly mixed and sieved. To provide root and rhizosphere material, pots were seeded, each with 15 seeds. Corresponding unseeded pots provided non-rhizosphere control soil in order to avoid the influence of roots which often completely permeated the soil in seeded pots. The pots were placed in the greenhouse and were watered as required. No water was supplied during the 24 hours prior to sampling, in order to facilitate removal of excess soil from the roots when plants were removed from the pots.

The following soils were selected for study: (*a*) Woburn light sandy loam (Stackyard field), pH 5.6; (*b*) Rothamsted clay loam (Pastures field), pH 6.6; (*c*) Sewell chalk (collected near Sewell, Beds.), pH 7.8; Barnet clay loam (from Bignall Nurseries, Barnet, Herts.), pH 6.8.

Plants were carefully removed from the pot and were shaken individually to remove the bulk of the soil adhering to their roots. Any aggregates of soil remaining on rootlets were dislodged. Roots were snipped off at the crown and dropped into a screw-capped bottle containing 100 ml. sterile water, sufficient root material being used to provide approximately 1 g. of rhizosphere soil. This initial dilution was shaken vigorously by hand for 5 minutes. Serial 10-fold dilutions were made before the heavier particles could settle. Six replicate plates were prepared from the 1:10,000 and 1:100,000 dilutions, 1 ml. of the appropriate dilution being mixed with the plating medium, previously melted and cooled to 45° C.

The roots were removed from the original dilution, thoroughly washed, and the wash water returned to the bottle. The contents of the bottle were evaporated to dryness at 85° C. to determine the oven-dry weight of the rhizosphere soil for estimation of the number of fungi per gram.

The roots from which a rhizosphere sample had been prepared were subjected to a serial-wash treatment similar to that described by Harley and Waid (3). They were cut into 2-in. lengths and, after removal of lateral rootlets, were transferred to 25 ml. screw-capped bottles, each containing 10 ml. of sterile water. Not more than 10 root lengths were placed in each bottle which was shaken by hand for 1 minute, and the wash water was decanted off and replaced with fresh sterile water. This procedure was repeated until 20 successive changes of water were completed. After every four changes the roots were transferred to a fresh 10 ml. sterile water blank in order to avoid carry-over of sediment. The washed roots were then removed to sterile water in a Petri plate where they were cut into 0.5 cm. pieces before they were placed on the surface of agar in previously poured plates.

To prepare a non-rhizosphere sample the surface quarter-inch of soil in an unseeded pot was removed with a flamed spatula and approximately 1 g. of soil, taken from the upper 2 inches in the pot, was transferred to a 100 ml. sterile water blank. The procedure was then identical with that described for the rhizosphere sample.

Peptone dextrose agar (7) containing rose bengal and chlortetracycline* was used for enumeration and primary isolation of fungi. Rose bengal was added at the rate of 33.3 mg./l. after the medium had been heated to melt the agar. A freshly prepared sterile solution of chlortetracycline was added to the cooled, sterile medium to give a concentration of 30 mg./l. immediately before plates were poured. The plates were incubated at room temperature (16°–22° C.). Fungal counts were made on rhizosphere and control plates after 6 days' incubation, and as many fungi as possible were isolated on potato dextrose agar slants from representative plates for subsequent identification. Similarly, fungal colonies, approximately 100 cultures per sample, were isolated from washed root pieces after 10 days' incubation. All plates were retained for at least 3 weeks so that certain slowly developing fungi could be isolated.

Results

Influence of Plant Age

Platings for fungi were made from healthy wheat roots at four intervals from the seedling stage to maturity. Two soils used in the experiment, Woburn light sandy loam and Barnet clay loam, were known to support healthy growth of wheat although they differed in origin, texture, reaction, and fertility. The numbers of fungi in rhizosphere and non-rhizosphere soil, as determined by the dilution method, are shown in Fig. 1.

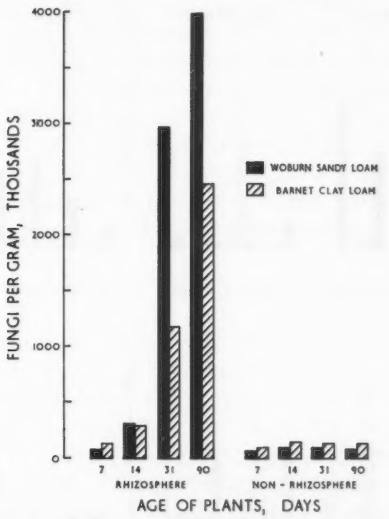

FIG. 1. Stimulation of fungi in the rhizosphere of wheat in two different soils.

*Chlortetracycline, previously recommended by Cooke (1) as a bacterial inhibitor in fungal isolation media, provided complete control of streptomycin-resistant bacteria which were found to be abundant in rhizosphere soil and which appeared on fungal plates containing streptomycin as the antibacterial substance (7). The sample of chlortetracycline (Aureomycin) was kindly supplied by Lederle Laboratories Division, Cyanamid Products Ltd., Bush House, Aldwych, London, W.C. 2.

Total counts of fungi in rhizosphere soil increased markedly with plant age, highest counts being recorded in samples from the light sandy soil. The numbers in non-rhizosphere control samples from both soils remained relatively constant.

The relative proportions of the predominant types of fungi on the root surface, in the rhizosphere and in non-rhizosphere controls are shown in Fig. 2. Various other genera represented in the samples usually occurred in very low numbers or were isolated only occasionally. In general, the pattern of fungal distribution was similar for the two soils. Species of *Fusarium* were abundant

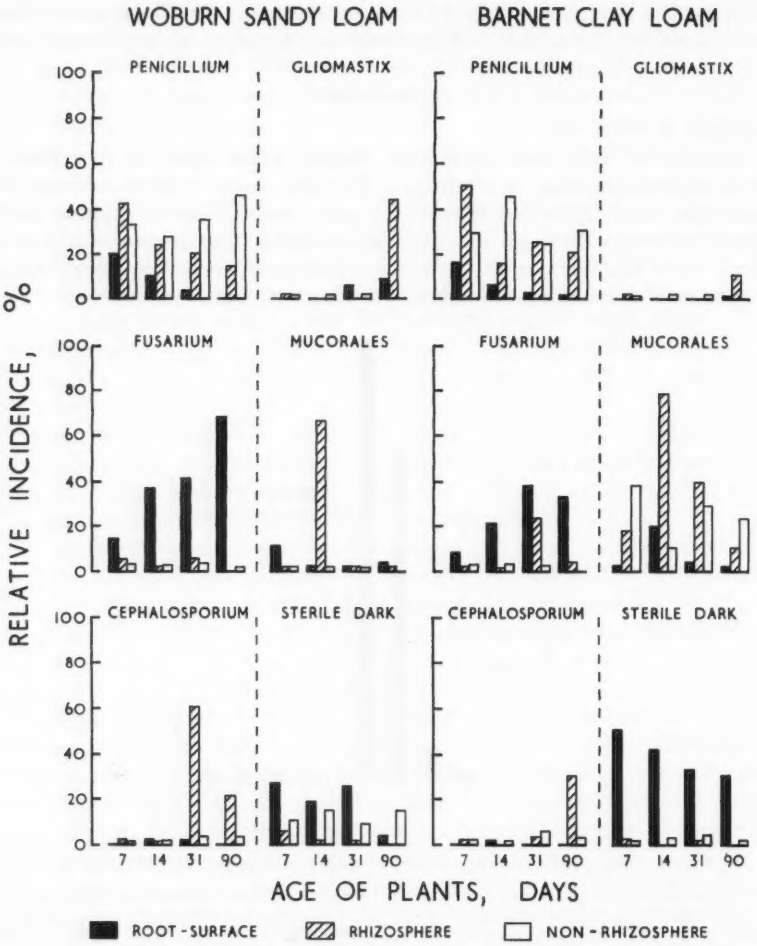

FIG. 2. Comparison of predominant root surface and rhizosphere fungi of wheat grown in two different soils.

on the roots, their relative incidence increasing with plant age, but were usually found in low numbers in rhizosphere and non-rhizosphere samples. *F. oxysporum* accounted for 78 and 67% of the total fusaria isolated from roots from the sandy loam and the clay loam, respectively. Unidentified sterile dark fungi (non-sporing on potato dextrose agar) were isolated frequently from the washed roots, higher proportions being recorded from the roots of the clay loam series than from the sandy loam series. Many of these dark fungi appeared at the cut ends of the root pieces, suggesting that they arose from internal root tissues rather than from the root surface. Their numbers, however, were comparatively low in rhizosphere and control soil.

Species of *Penicillium* were common in rhizosphere and non-rhizosphere soil throughout the experiment. Their activity on the roots appeared to be confined mainly to the seedling stage since the relatively high proportions of these fungi obtained from the roots 7 days after seeding were found to decline in subsequent samples.

Although *Cephalosporium* spp. appeared in highest numbers in rhizosphere samples from the sandy loam a month after seeding, a similar increase occurred in corresponding samples from the clay loam only when the plants had reached maturity. *Gliomastix* spp. also were found to increase in the rhizosphere of mature plants, particularly in the sandy soil. The proportions of both *Cephalosporium* and *Gliomastix* were low on the roots and in non-rhizosphere soil.

In the clay-soil series the Mucorales, represented by the genera *Absidia*, *Mucor*, *Mortierella*, and *Zygorhynchus*, were common in rhizosphere and non-rhizosphere samples. The marked increase in their proportions on washed roots and in rhizosphere soil noted 2 weeks after seeding was due mainly to a rise in the proportions of *Mucor* and *Zygorhynchus* at that time. Species of the Mucorales were generally scarce in samples from the sandy loam series; however, a sharp increase in numbers, due almost entirely to *Zygorhynchus*, also occurred in the rhizosphere 2 weeks after seeding, but the effect did not persist in later samplings.

Influence of Soil Type

Red clover and wheat plants grown in the three soils, Woburn light sandy loam, Rothamsted clay loam, and Sewell chalk were sampled 5 weeks after seeding. The numbers and types of fungi occurring in rhizosphere and non-rhizosphere soil were determined by means of the dilution method. Root segments subjected to serial washing were plated to provide information on the fungi harbored by them. The estimated numbers of fungi in rhizosphere soil and in corresponding control soil are shown in Table I.

Whereas soil type did not appear to influence total numbers of fungi in the rhizosphere of red clover, distinct differences were noted in that of wheat, highest counts being recorded for the light sandy soil.

Although a comparatively wide range of fungal types was obtained from the various samples examined, only the predominant forms are shown in Table II.

TABLE I

EFFECT OF SOIL TYPE ON NUMBERS OF FUNGI IN THE RHIZOSPHERE OF RED CLOVER AND WHEAT

Soil	Sample	Thousands per g.*	
		Red clover	Wheat
Sandy loam (acid)	Rhizosphere	816.7	4044.9
	Non-rhizosphere	127.4	120.8
Clay loam (neutral)	Rhizosphere	820.6	1136.4
	Non-rhizosphere	128.8	122.7
Chalk (alkaline)	Rhizosphere	744.7	1806.6
	Non-rhizosphere	132.0	130.4

*Averages, each of six replicate plates (1:10,000).

Those which occurred irregularly or infrequently are not included. It is evident that soil type exerted a distinct effect on the distribution of certain fungi, not only in the soil but also on the roots and in the rhizosphere. Species of *Mortierella* and *Gliomastix* were found to be more prevalent in the alkaline chalk soil than in either the acid sandy loam or the nearly neutral clay loam, whereas *Trichoderma* spp. were found only in samples from the two latter soils. The apparent stimulation of *Trichoderma* and *Cephalosporium* only in the rhizosphere of wheat grown in the acid sandy loam, and of *Gliocladium* in the rhizosphere of wheat from the alkaline chalk soil further attests to the influence of soil type. However, the apparent lack of similar stimulatory effects in red clover suggests that other factors, such as plant type and stage of plant growth, as well as soil type, affect the nature of the fungal flora associated with roots.

Species of *Penicillium* were abundant in rhizosphere and non-rhizosphere samples from both the sandy and the clay soils, but were comparatively rare in corresponding samples from the chalk soil. The latter soil appears to be unusual in view of the results of Nicholls (8), who found penicillia to be particularly abundant, both in numbers and species, in eight chalk soils obtained from different localities. Nevertheless, it is generally considered that *Penicillium* spp. are usually most common in acid soils (5, 14).

Perhaps the most interesting feature of these experiments was the influence of soil type on the predominance of specific fungi on the root surface, a phenomenon which would have been overlooked by exclusive use of the dilution plate method. A striking difference was noted between the acid sandy soil and the alkaline chalk soil in the proportions of *Fusarium* and *Cylindrocarpon* spp. In the former soil, species of *Fusarium* (mainly *F. oxysporum*) were predominant on the root surface, particularly on that of red clover, whereas species of *Cylindrocarpon* were almost entirely absent. In the latter soil, in contrast, species of *Cylindrocarpon* predominated on the root surface, the fusaria being comparatively rare. The effect in the nearly neutral clay soil appeared to be somewhat intermediate between that of the acid and alkaline soils since both *Fusarium* and *Cylindrocarpon* were found to occur on the root surface, in approximately equal proportions in the case of clover.

TABLE II

EFFECT OF SOIL TYPE ON PREDOMINANT ROOT-SURFACE AND RHIZOSPHERE FUNGI

Plant	Genus	Relative incidence, %								
		Root-surface			Rhizosphere			Non-rhizosphere		
		(A)*	(B)	(C)	(A)	(B)	(C)	(A)	(B)	(C)
Red clover	*Mortierella*	0.9	–	4.3	1.4	0.6	25.0	7.0	1.0	26.1
	Cephalosporium	–	–	0.7	–	1.7	8.8	–	6.1	10.1
	Trichoderma	–	–	–	3.6	1.1	–	3.0	6.1	–
	Penicillium	3.6	4.7	–	75.5	62.3	2.6	47.0	56.1	1.7
	Gliocladium	–	12.1	–	2.2	14.4	10.0	4.0	5.1	10.9
	Gliomastix	–	–	–	–	0.6	7.5	–	–	17.6
	Fusarium	84.7	35.6	3.5	2.9	–	–	1.0	1.0	–
	Cylindrocarpon	1.8	33.6	63.1	–	1.1	1.3	1.0	–	0.8
Total cultures examined		111	149	141	139	180	80	100	98	119
Wheat	*Mortierella*	–	–	0.8	1.5	1.7	18.8	2.0	3.0	25.5
	Cephalosporium	–	–	4.8	28.6	1.7	–	2.0	3.0	2.0
	Trichoderma	3.9	–	–	26.3	1.7	–	12.0	–	–
	Penicillium	1.0	3.7	–	30.1	68.6	1.2	28.0	45.0	2.9
	Gliocladium	–	1.9	4.0	3.8	9.1	40.0	1.0	6.0	2.9
	Gliomastix	2.9	5.6	6.4	4.5	2.5	9.5	2.0	–	19.6
	Fusarium	48.0	28.0	0.8	1.5	0.8	–	2.0	4.0	–
	Cylindrocarpon	–	6.5	29.6	–	–	–	–	1.0	1.0
Total cultures examined		102	107	125	133	121	85	100	100	102

*Soils: (A) sandy loam (acid); (B) clay loam (neutral); (C) chalk (alkaline).

Discussion

In spite of known limitations of technique, the present studies have shown that plant roots exert a marked influence on soil fungi in their vicinity. Quantitative studies revealed a more pronounced rhizosphere effect in a relatively infertile sandy soil than in a more fertile clay soil. Such differences suggest that root excretions are more effective as microbial stimulants under conditions of limited available substrate. The differences in fungal numbers noted between wheat and clover rhizosphere samples perhaps reflect a greater capacity for root excretion in the former than in the latter. However, a true comparison of the two crops cannot be made inasmuch as wheat, by virtue of its rate of development, had reached a more advanced state of maturity than the clover, even though the seedlings sampled were of the same age.

Both plant age and soil type appear to affect the nature of the fungal population associated with roots. The relatively high proportions of fungi such as the penicillia and certain species of the Mucorales found on the root surface and in the rhizosphere of wheat in the early stages of development may well coincide with root excretion of specific substances during that period. In this connection, Rovira (9) reported the presence of fructose and glucose in samples of root exudate from both peas and oats which were grown in sterile sand culture for 10 days, but not in similar samples obtained at 20 days. Although excretion of such sugars would be expected to stimulate various fungi, particularly species of the Mucorales, further information correlating root excretion of specific substances with specific fungi or other microorganisms is required on a wide range of plants before generalizations may be made.

The predominance of *Fusarium* and/or *Cylindrocarpon* (depending upon soil type) on the roots of red clover and wheat without apparent injury to the plants suggests that such fungi may be normal root surface inhabitants. However, as many of these fungi are known to be potential pathogens they might be expected to assume a parasitic role under conditions which reduce plant vigor. Presumably, the observed effects in the different soils tested were due largely to the influence of soil reaction. According to Garrett (2) the *Fusarium* wilts, caused by various biologic forms of *F. oxysporum*, tend to be favored by acid soil conditions. Katznelson and Richardson (6) obtained evidence that certain fungi of pathogenic capability, including *Cylindrocarpon*, were more sensitive to an acid soil reaction than common saprophytic forms.

Various sterile dark fungi isolated regularly from washed roots even as early as 7 days after seeding appeared to arise mainly from internal tissues. Recently, Waid (13) reported that such fungi were abundant in the inner and outer cortex of rye-grass roots and believed them to be early colonizers of those tissues. Little is yet known about the influence of such fungi on plant growth but it is possible that they may be concerned with the plant in a relationship approaching symbiosis. Further information on fungi associated with roots of healthy plants under a wide range of environmental conditions seems necessary in order to obtain a more complete picture of their distribution and activities in relation to the nutrition and general welfare of plants.

Acknowledgment

The author wishes to thank Dr. H. G. Thornton, formerly Head of the Soil Microbiology Department, Rothamsted Experimental Station, for his advice and interest during the course of this work.

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HISTOPATHOLOGY OF GRANULOSIS VIRUSES IN INSECTS[1]

F. T. BIRD[2]

Abstract

A histopathological study of granulosis virus diseases of the spruce budworm, *Choristoneura fumiferana* Clem., the larch budworm, *Eucosma griseana* Hbn., and the imported cabbage worm, *Pieris rapae* Linn., has shown that the viruses may infect fat, hypodermal, tracheal matrix, and blood cells. The viruses multiply and develop within the cytoplasm of these cells appearing first as spheres and then as rods. Finally each rod becomes occluded by protein to form a "granule".

Introduction

Two of the best-known types of virus diseases affecting insects are nuclear polyhedrosis and granulosis. The polyhedrosis diseases are characterized by the formation of crystal-like protein bodies, about 0.5 to 3 μ in diameter, within the nuclei of hypodermal, tracheal matrix, fat, blood, and, in advanced cases of infection, other cells of the insect's body. Each polyhedron contains many virus particles in the form of rods, 20 to 70 mμ in diameter and about 200 to 400 mμ in length, and frequently some in the form of spheres. A study of the different forms of virus particles obtained by dissolving pure polyhedra in weak alkali led Bergold (2) to propose a life cycle beginning with spheres and ending with rods. Bird and Whalen (5) demonstrated that the rods appear "chiefly on exposed surfaces of strands of chromatin", and later Bird (4) demonstrated masses of spherical bodies in the infected nucleus as well as rods attached to strands of chromatin. It was suggested that a developmental cycle began at the time of the attachment of rods to chromatin. Further support for this thesis has been obtained in recent studies of a polyhedrosis of the spruce budworm, *Choristoneura fumiferana* Clem. In this disease, bundles of rods instead of single rods develop in the nucleoplasm but only single rods are found attached to the chromatin. Polyhedra are formed by the accumulation of protein around single rods or bundles of rods depending on the particular virus disease (4, 6, 7).

Much less is known concerning the histopathology of the granulosis diseases. Heretofore, it has not been proved whether the viruses causing granulosis multiply and develop in the nucleus, in the cytoplasm, or in both. Also, different tissues have been reported to be susceptible in different insect species. All investigators agree, however, that the fat tissue is involved (8, 10, 11, 12, 14, 15, 16). Some authors (10, 11, 12, 14, 16) report the infection of hypodermis as well as fat, some (14) report the infection of fat, hypodermis, and tracheal matrix, and one author (1) has found infected blood cells. Thus granulosis viruses have been reported as developing in the same tissues as the polyhedrosis viruses, although not all the different tissues have been found infected in any one insect species.

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Paillot (10) believed that the virus causing granulosis of *Pieris brassicae* Linn. multiplied in the cytoplasm but not in the nuclei of fat and hypodermal cells. He later (11) described two granuloses of *Euxoa segetum* Schiff., in one of which he reported that granules appeared first in the cytoplasm while in the second he believed that the granules developed in the nucleus as well as in the cytoplasm. Paillot (12) also described what he believed to be a third granulosis of the same insect in which the granules appeared only in the nucleus. Bergold (1) demonstrated in dark-field light microscope pictures "blasenformige Gebilde" filled with granules in the cytoplasm of blood cells of *Choristoneura murinana* Hbn. Steinhaus and Thompson (14) in describing a granulosis of *Junonia coenia* Hbn. stated: "The granules possibly originate within the nucleus and sooner or later accumulate in the cytoplasm." Hughes and Thompson (8) believed that the granules of the disease affecting *Sabulodes caberata* Guenée formed in the nucleus. They pointed out that "it is possible that some reports of granuloses which arise in the cytoplasm may have resulted from observations made after the infection had progressed too far to distinguish between nucleus and cytoplasm". Tanada (16), Martignoni (9), and Smith and Rivers (13), in investigations of granulosis diseases of *Pieris rapae*, *Eucosma griseana*, and *P. brassicae*, respectively, all report the development of the viruses within the nuclei of susceptible cells.

Virus particles associated with granuloses were first demonstrated by Bergold (1) and later by Steinhaus, Hughes, and Wasser (15), Hughes and Thompson (8), Tanada (16), Bergold (3), and Smith and Rivers (13). Each granule, with dimensions about 0.5×0.15 μ, usually contains one virus particle though occasionally two occur. They are either rods similar in size to those found in polyhedra, or spheres. The life-cycle theory proposed by Bergold (2), wherein rods develop from spheres, applies to the granuloses as well as to the polyhedroses.

A histopathological study of a granulosis disease affecting the spruce budworm, *C. fumiferana*, forms the major part of the present communication. The virus particles were first demonstrated by Bergold (3). Two other granuloses were also studied: one affecting the larch budworm *E. griseana*, the virus particles of which were isolated by Martignoni (9), and the second affecting *P. rapae*, the virus particles of which were isolated by Tanada (16).

Methods

Spruce budworm larvae were collected from hibernacula spun in the field and reared in the laboratory.* They were infected, while in the fourth instar, with granulosis virus by allowing them to feed on infected fat tissue of freshly killed, diseased larvae macerated in distilled water. A drop of the suspension was placed on a glass slide and each larva was observed under a dissecting microscope and not removed until it had devoured some of the suspension. *Budworm larvae are quite cannibalistic* and usually sought out the solid pieces

*Miss A. R. McMorran supplied most of the spruce budworm larvae.

of tissue. Larvae hatching from eggs of *E. griseana*† were infected while in the fourth instar by placing a drop of a suspension of partially purified granules‡ suspended in distilled water on their mouth parts. *P. rapae* were reared on cabbage out of doors. The cabbages were infested naturally. The larvae also were infected in a late instar, by placing a drop of a suspension of partially purified granules, obtained from diseased larvae infected with a native virus, on their mouth parts.

Blood cells of healthy and diseased larvae were examined under the dark-field and phase-contrast microscopes simply by placing a drop of blood, obtained by cutting the tip off a proleg, on a slide and covering with a cover glass. For purposes of photography, a smear was prepared and allowed to dry. A drop of water was then placed on the smear and covered with a cover glass. There is little movement of the contents of the cells during photographing but the cells are flattened and granule-filled vesicles are ruptured by this treatment. Some smears were fixed, after drying, and stained with Giemsa's or with Wright's blood stain.

Blood cells from healthy and diseased larvae were prepared for thin sectioning and electron microscope study as follows: Blood oozing from cut prolegs of larvae was allowed to drop into 2% osmium tetroxide buffered at pH 7. After 2 hours' fixation, the suspension was centrifuged at 8000 r.p.m. for 5 minutes and the supernatant discarded. The pellet was dehydrated in an alcohol series, centrifugation being necessary at each alcohol change, and embedded in three parts of butyl methacrylate and one part methyl methacrylate. Sections were cut with a Sjöstrand microtome, using a glass knife, and placed on carbon films for examination under the electron microscope.

Fat, hypodermal, tracheal matrix, and other tissues were studied in stained sections under the light microscope and in thin sections under the electron microscope. Stained sections were prepared by fixation of the tissues in Bouin's fluid followed by staining in Heidenhain's iron haematoxylin. Nuclear material containing desoxyribonucleic acid was identified in sections exposed to the Feulgen reaction. For thin sectioning, small pieces of tissue were fixed in 2% osmium tetroxide and embedded in methacrylate in the same manner as described for blood cells.

Results

The granulosis viruses affecting *C. fumiferana* and *P. rapae* develop in the fat, hypodermal, tracheal matrix, and blood cells. Only the fat cells of *E. griseana* have been studied so far and although it is known that the granulosis affecting this insect develops in the fat cells, it has not been determined whether the virus also develops in hypodermal, tracheal matrix, and blood cells. In *C. fumiferana*, which has been studied most intensively, the fat is the first tissue infected, then hypodermis and tracheal matrix. The blood cells are last to be infected.

†Egg masses of *E. griseana* were kindly supplied by Prof. Dr. P. Bovey, Entomologisches *Institut, Technische Hochschule, Zurich,* Switzerland.

‡The virus suspension was obtained from M. E. Martignoni, then associated with Eidg. Anstalt für das forst Versuchwesen, Switzerland.

Dark-field and phase-contrast studies of blood cells of *C. fumiferana* and *P. rapae* show that the virus granules form only in the cytoplasm. Fig. 1 shows in dark field two blood cells, one of which is in an advanced stage of infection and contains a large highly refractive mass of virus granules in the cytoplasm. A phase-contrast picture of granulosis-infected blood cells is shown in Fig. 2. The small, black-appearing virus granules appear only in the cytoplasm. In this photograph the cells are ruptured and the granules are scattered. We were unable to demonstrate the granules in fixed and stained preparations of blood cells.

Electron micrographs of thin sections of blood cells are shown in Figs. 3 and 4. They show very clearly that the capsule-shaped virus granules are formed in the cytoplasm. An early stage in the development of granulosis in blood cells of *C. fumiferana* is shown in Fig. 5, in which groups of virus particles appear chiefly in the form of spheres. Fig. 6 is a photograph of a later stage when protein has surrounded individual particles to form granules.

A light microscope photograph showing granulosis-infected fat, tracheal matrix, and hypodermis is shown in Fig. 7. Infected tissues contain patches of dull gray, homogeneous, granular material crisscrossed with dark-blue strands when stained with Heidenhain's iron haematoxylin. The virus granules, which make up the granular masses, are not resolved in sections photographed under the light microscope and it is extremely difficult to distinguish these masses, the outer limits of which appear rather smooth in outline, from nuclei. The Feulgen reaction gives a very slight positive result for these granular masses and a strong positive result for the chromatin in nuclei. Intact nuclei may therefore be demonstrated in fairly advanced cases of granulosis infection by using the Feulgen reaction.

Dark-field and phase-contrast microscopy have more limited use with fat, hypodermis, and tracheal matrix than with blood cells. These tissues are multicellular and it is difficult to obtain a preparation sufficiently thin to distinguish individual cells. Individual virus granules may be resolved in squash preparations of these tissues, however, using either dark-field or phase-contrast microscopy. Normal ganglia and normal hypodermis contain granular material only slightly larger and of similar refractivity, and some experience is necessary to distinguish virus granules from this material. A very useful combination of lenses in the diagnosis of squash preparations is a dark-field condenser and low-power (10×) objective. Patches of light-blue refracting, infected tissue stand out clearly from the pale healthy tissue.

Electron microscope studies of thin sections of granulosis-infected fat tissue of *C. fumiferana*, *E. griseana*, and *P. rapae* have shown a similar infection process in these three diseases. Groups of virus particles, free or enclosed by protein, may occur throughout the cytoplasm of fat cells as shown for *E. griseana* in Fig. 8. What was considered in the study of blood cells to be a very early stage in virus development, i.e. when the particles consisted mostly of free spheres, has been observed only once in fat tissue. A group of spheres was found in the cytoplasm of fat tissue of *C. fumiferana* near the nuclear

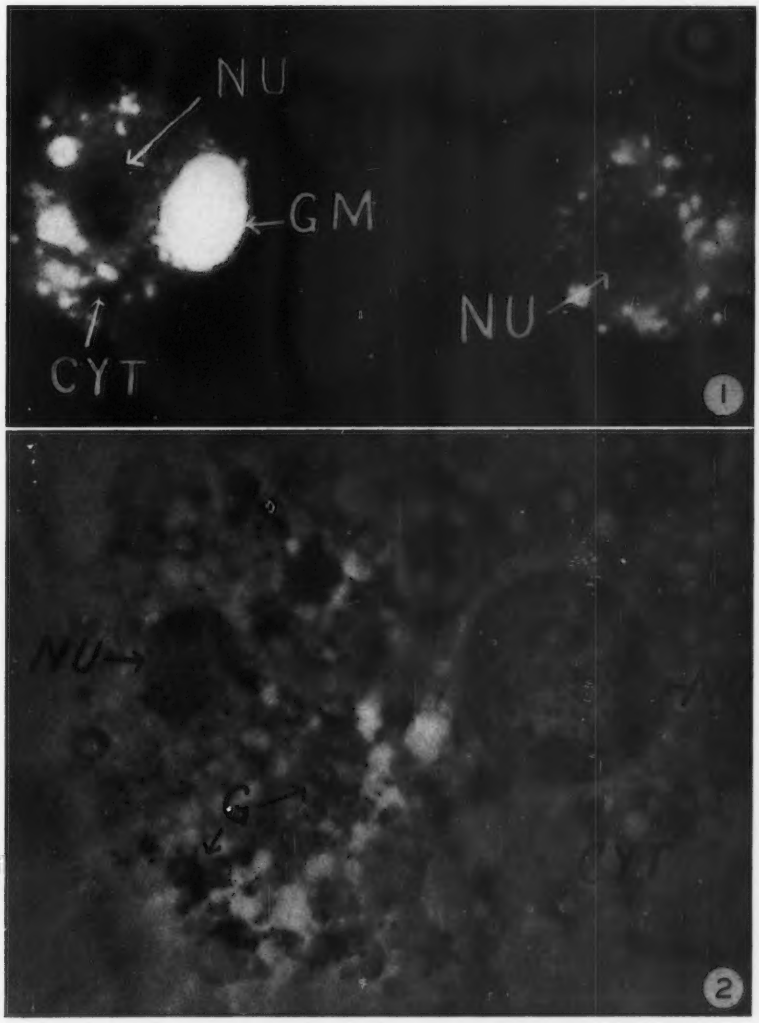
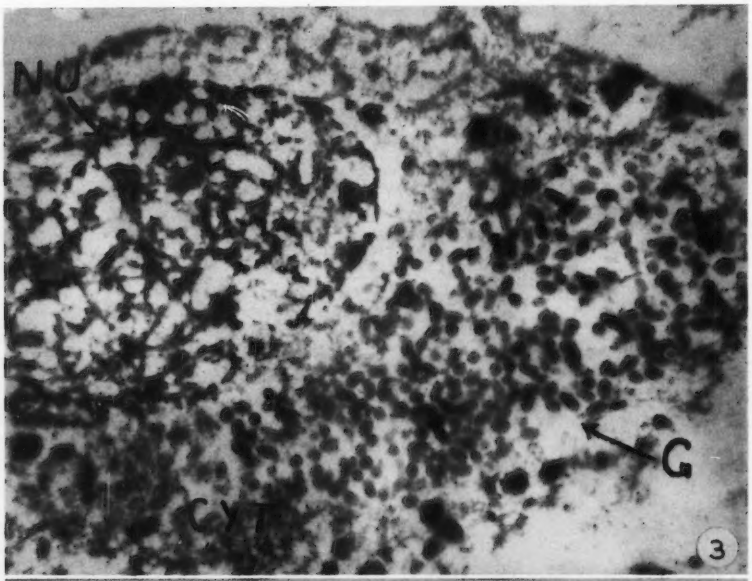
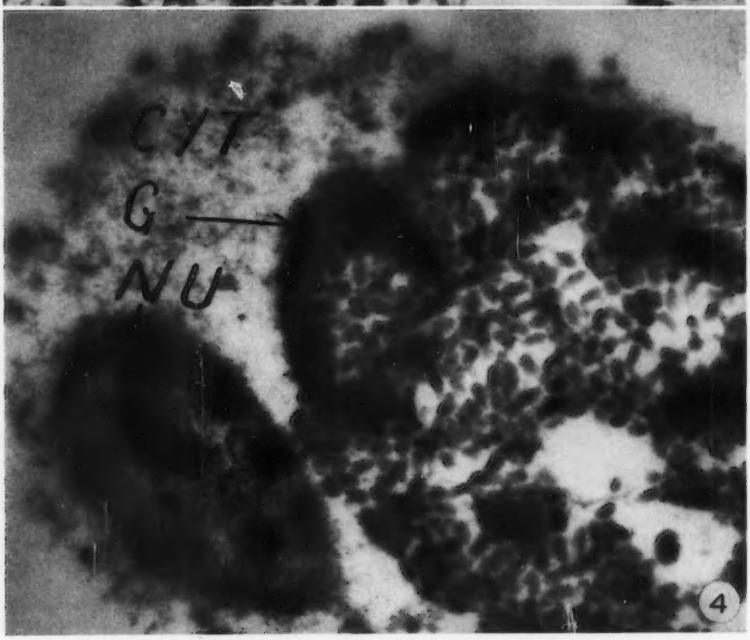


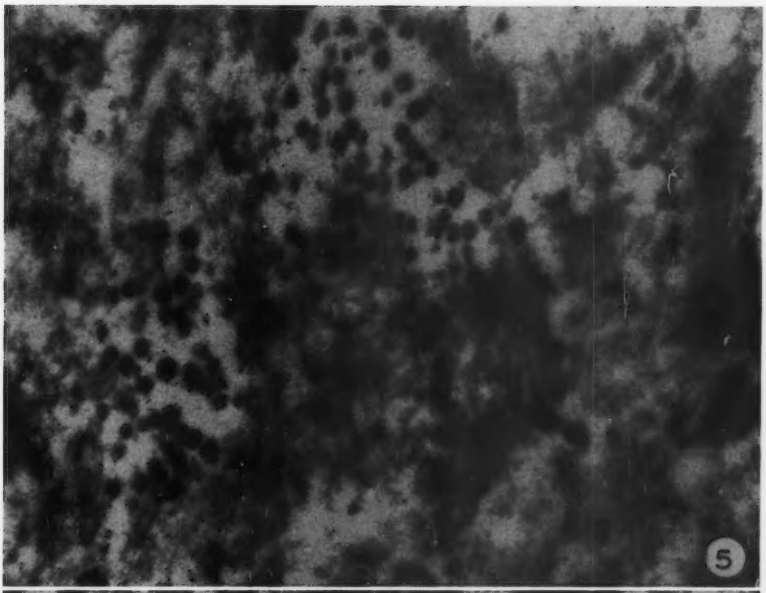
FIG. 1. Dark-field light microscope photograph of granulosis-infected blood cells of *C. fumiferana* Clem. Blood cell at left contains a large mass of very refractive granules in the cytoplasm. GM, granular mass; NU, nucleus; CYT, cytoplasm. ×1800

FIG. 2. Phase-contrast light microscope photograph of granulosis-infected blood cells of *C. fumiferana* Clem. showing infection and individual granules in the cytoplasm of one cell. G, granules; NU, nucleus; CYT, cytoplasm. ×2200





FIGS. 3 and 4. Electron microscope photographs of thin sections of granulosis-infected blood cells of *C. fumiferana* Clem. showing granules in the cytoplasm of the cells. NU, nucleus; G, granules; CYT, cytoplasm. ×10,000



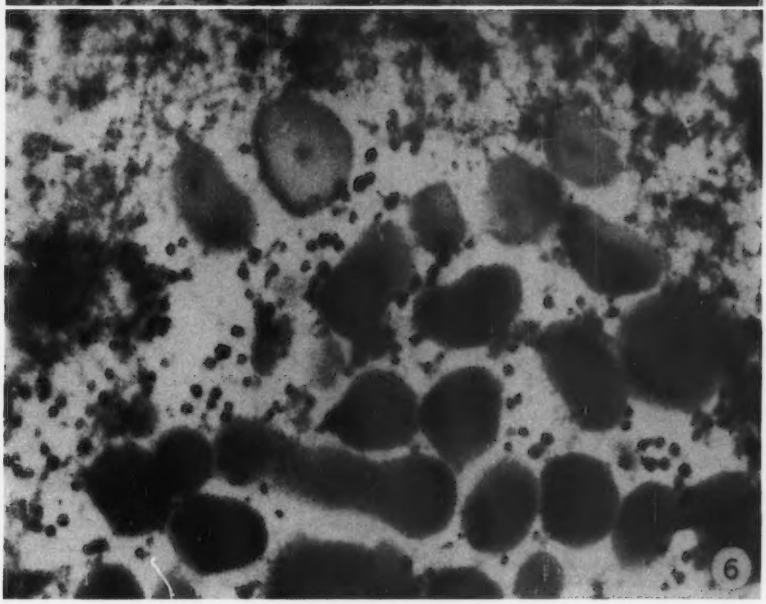
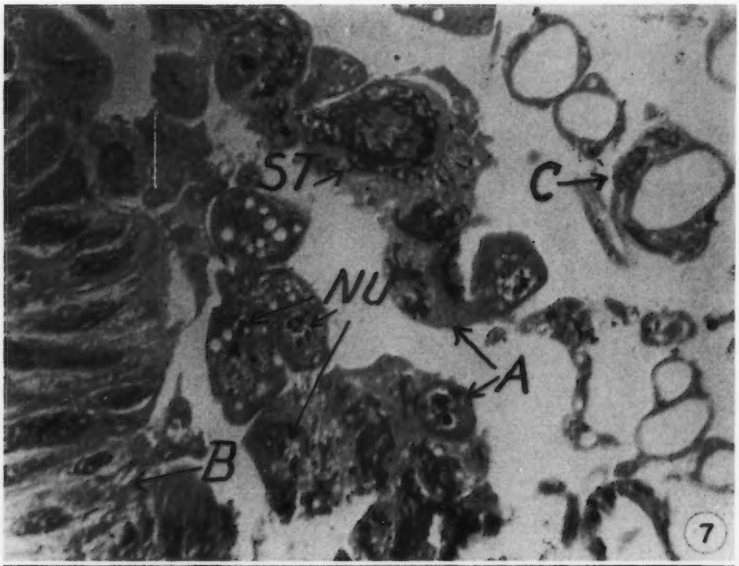


FIG. 5. Electron microscope photograph of a thin section of granulosis-infected blood cell of *C. fumiferana* Clem. showing a group of free, mostly spherical virus particles in the cytoplasm. ×40,000

FIG. 6. Electron microscope photograph of a thin section of a granulosis-infected blood cell of *C. fumiferana* Clem. showing free virus particles and particles surrounded by protein to form granules. ×40,000



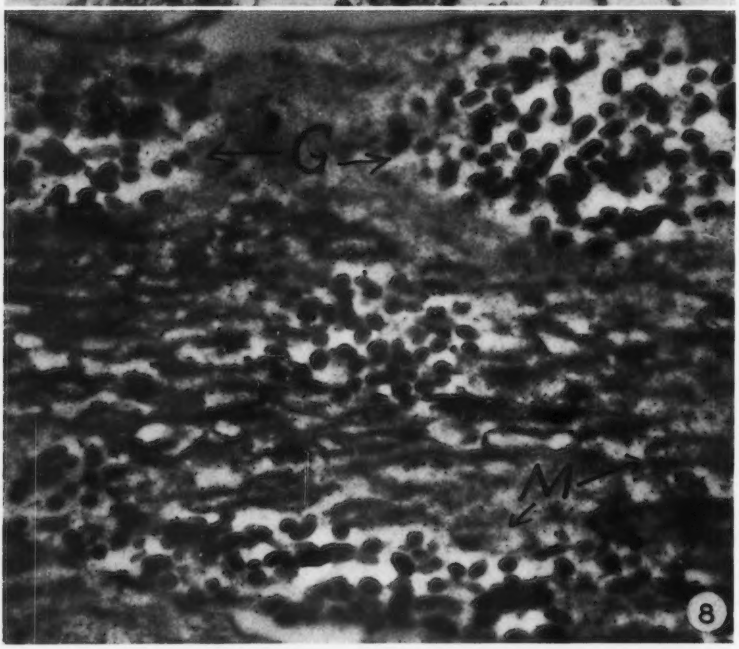


FIG. 7. Light microscope photograph of a section stained with Heidenhain's iron haematoxylin showing granulosis infection of (A) fat, (B) hypodermis, and (C) tracheal matrix. Infected parts of cells are crisscrossed with dark-blue-stained strands. Several apparently normal nuclei are present. NU, nucleus; ST, strands.

FIG. 8. Electron microscope photograph of a thin section of granulosis-infected fat of *E. griseana* Hbn. showing several groups of granules in the cytoplasm. G, granules; M, mitochondria. ×10,000

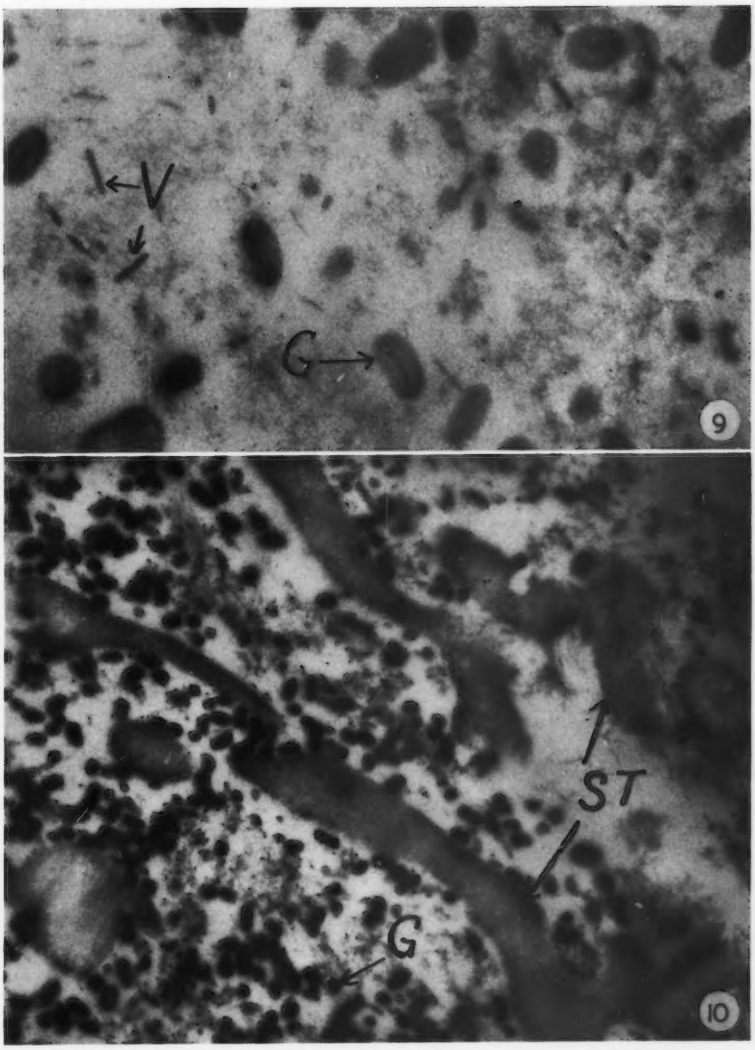


FIG. 9. Electron microscope photograph of a thin section of granulosis-infected fat of *C. fumiferana* Clem., showing (V) free virus particles and (G) granules containing single virus particles. ×40,000

FIG. 10. Electron microscope photograph of a thin section of granulosis-infected fat of *C. fumiferana* Clem., showing strands of dense material that are probably analogous to dark-stained strands shown in Fig. 7. ST, strands; G, granules. ×10,000

PLATE VI

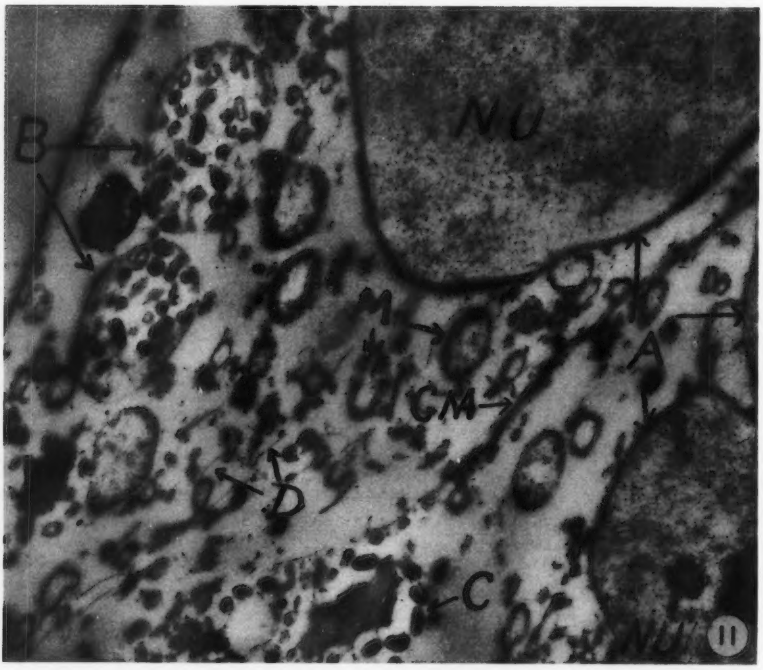


FIG. 11. Electron microscope photograph of a thin section of advanced granulosis-infected fat of *C. fumiferana* Clem. showing (A) parts of intact nuclei; (B) accumulation of granules in large vesicles; (C) scattered granules in cytoplasm; and (D) long filaments. NU, nucleus; M, mitochondria; CM, cell membrane. ×10,000

membrane. Fig. 9 shows free rods and granules in the cytoplasm of fat tissue of *C. fumiferana*. The rods appear concentrated along denser parts of the tissue not unlike the condition in polyhedroses where rods are attached to strands of chromatin. It has not been determined whether this dense material is analogous to the strands observed in sections stained with haematoxylin and examined under the light microscope. In very advanced cases of infection what appears to be a residue of compact material in the form of strands is present; this perhaps is the material observed under the light microscope (Fig. 10). As with blood cells, no evidence has been obtained to indicate that the viruses develop in the nuclei of fat cells. The nuclei show degenerative changes in that some of the nuclear material clumps together and some becomes finely granulated, but an intensive examination of nuclei in tissue in various stages of granulosis infection has failed to reveal evidence of virus development within them. Fig. 11 shows parts of three intact nuclei in a section of fat tissue of *C. fumiferana* in an advanced stage of granulosis infection. There are many virus granules in the cytoplasm but none in the nuclei. It is of interest to note the accumulation of virus granules in two large vesicles in the cytoplasm. One can also observe in this photograph long filaments about the same diameter and density as free virus rods. The significance of these filaments has not been determined.

Conclusions

Granulosis viruses of the spruce budworm, *C. fumiferana* Clem., and the cabbage worm, *P. rapae* Linn., develop in the cytoplasm of fat, tracheal matrix, hypodermis, and blood cells. A study of the fat tissue only of the larch budworm, *E. griseana* Hbn., has also shown the development of the virus in the cytoplasm. This conclusion is in opposition to that of Tanada (10), who previously studied the granulosis of *P. rapae*, and to that of Martignoni (9), who studied the granulosis of *E. griseana*, as well as to the conclusion reached by many others that granuloses develop in the nucleus. The virus granules about 0.5×0.15 μ in size cannot be detected in stained sections examined under the light microscope, the technique used by most investigators, and it is very easy to confuse cytoplasmic and nuclear elements in advanced stages of infection. Phase-contrast and dark-field microscopy of blood cells, however, show very clearly that groups of the granules are formed in the cytoplasm, and electron microscopy of blood, fat, and other tissues has revealed various stages in the development of the viruses in the cytoplasm.

The development of granulosis in the cytoplasm appears to be similar to the development of polyhedrosis in the nucleus (4) in that virus particles go through a similar developmental cycle. The virus appears in the form of spheres that develop into rods which become surrounded by protein to form the granules. Unlike the polyhedra which may contain many single rods (4) or bundles of several rods (3, 7), each granule contains, except for an occasional bundle of two rods, a single rod. Although not encountered in this study, Bergold (2) has shown that some granules contain immature spherical forms of the virus.

Acknowledgments

Grateful acknowledgment is extended to Miss Ione A. Wingfelder for technical assistance and to members of the Forest Biology Laboratories at Sault Ste. Marie, Ontario, for their helpful criticisms of this paper.

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HYPHOMYCETOUS FUNGI: VOLUCRISPORA AURANTIACA N. GEN., N. SP.; V. ORNITHOMORPHA (TROTTER) N. COMB.; AND TRICELLULA CURVATIS N. SP., WITH THE GENUS TRICELLULA EMENDED[1]

R. H. HASKINS

Abstract

Three hyphomycetous fungi belonging to the Gloiosporae of the Staurosporae (Moniliales) are described and illustrated. The name *Volucrispora aurantiaca* n. gen., n. sp. is suggested for an organism characterized by conidiospores with one to five cells connected to a basal cell by narrow isthmuses or constrictions. The relationship of this fungus with *Titaea ornithomorpha* Trotter (1904) is discussed and the latter name should become *Volucrispora ornithomorpha* (Trotter) n. comb. The genus *Tricellula* van Beverwijk (1954) is emended to include *Tricellula curvatis* n. sp., which differs mainly in its characteristic spore shape from *Tricellula inaequalis* van Beverwijk, the type species of the genus *Tricellula*.

Introduction

During the isolation of fungi of the *Rhizophlyctis–Karlingia* group (Chytridiales) from soil samples, optically active orange-brown crystals (Fig. 1) appeared in one partially purified culture on uncoated cellophane submerged in water. The various fungus contaminants present in the mixed culture were isolated separately on Difco potato dextrose agar, and all were either discarded or set aside except for the one (PRL 1554) producing the orange-brown crystals. This fungus was propagated and studied extensively.

In this paper the morphology and development of the fungus is described, and its taxonomy and relationships with similar fungi discussed. The results of the chemical studies on its metabolic products will appear elsewhere.

The organism, a new species, is made the type of a new genus and the name *Volucrispora aurantiaca* is suggested. *Titaea ornithomorpha* Trotter should be removed from that genus and it should be named *Volucrispora ornithomorpha*.

To aid in the above study, cultures of the recently described *Tricellula inaequalis* from Europe, and from the United States of America, were obtained. All three organisms were studied at the same time and under identical conditions. The American isolate proved to be sufficiently different from the European isolate, the type for the genus, to warrant the naming of a new species, *Tricellula curvatis*, and the emendation of the generic description of *Tricellula* to permit its inclusion within the genus.

[1]Manuscript received February 4, 1958.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
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Volucrispora aurantiaca n. gen., n. sp.

Morphology and Development

The Spores

The fungus, PRL 1554, is characterized by three- to five-celled, essentially hyaline spores (pale orange-brown en masse), developing on agar plates in somewhat slimy masses from simple or compound conidiophores. The spore consists of two, usually three or four, sometimes five or more, cells—a basal cell bearing one apical cell and from none to several lateral cells (Figs. 2, 5, and 11). The basal cell varies from oblong to oval to rather triangular in shape, 4–6 μ long by 1–3 μ wide. On its distal end, which is often larger, the other cells are borne. The apical cell varies from obovate to acuminate in shape, 6–14 μ long by 1–3 μ wide. The similarly shaped lateral cells, 3–10 μ long by 1–3 μ wide, are borne on the "shoulders" of the basal cell and often take up a position reflexed from the apical cell. The over-all length of the spore varies from 10–20 μ. The over-all width varies greatly according to the number of lateral cells and the angles at which they are borne. The apical cell (and occasionally a lateral cell) is often slightly constricted around its central point.

The apical and lateral cells are each joined to the basal cell by means of an isthmus. This isthmus, in water cultures and on moist agar cultures, is very narrow and distinct (Figs. 2, 5, 6, 8, 11, and 13–17), occasionally over a micron in length. Often in younger cultures and in drier ones, the isthmus is short and broad (see Fig. 11) with no obvious crosswall separating the spore parts. While the spore easily breaks off from its sporophore, the apical and lateral cells remain firmly attached to the basal cell in spite of rough handling and extensive teasing over an agar surface.

In water cultures or on moist agar four-celled spores predominate (Fig. 5). In drier or less favorable conditions, the three-celled spores often are the more abundant (Figs. 2 and 3.).

Germination of Spores

The germination of the spore is preceded by a considerable swelling of its individual cells (Fig. 3). The basal cell is usually the first to germinate,

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FIGS. 1 to 10. *Volucrispora aurantiaca.*

FIG. 1. Orange-brown crystals in original cellophane–water culture. Edge of the piece of cellophane is visible on the left. × 40

FIG. 2. Three-celled spores. Note bird-like aspect of the spores, the narrow isthmuses joining the cells, and constrictions in several of the apical cells. Phase × 666

FIG. 3. Spores on cellophane on agar surface after 12 hours at 28° C. Individual cells have swollen considerably. Phase × 333

FIG. 4. Germinating spores on agar surface after 24 hours at 28° C. × 333

FIG. 5. Two-, three-, four-, and five-celled spores, lactophenol – cotton blue mount. Phase × 666

FIG. 6. Conidiophores with spores, lactophenol – cotton blue mount. Phase × 666

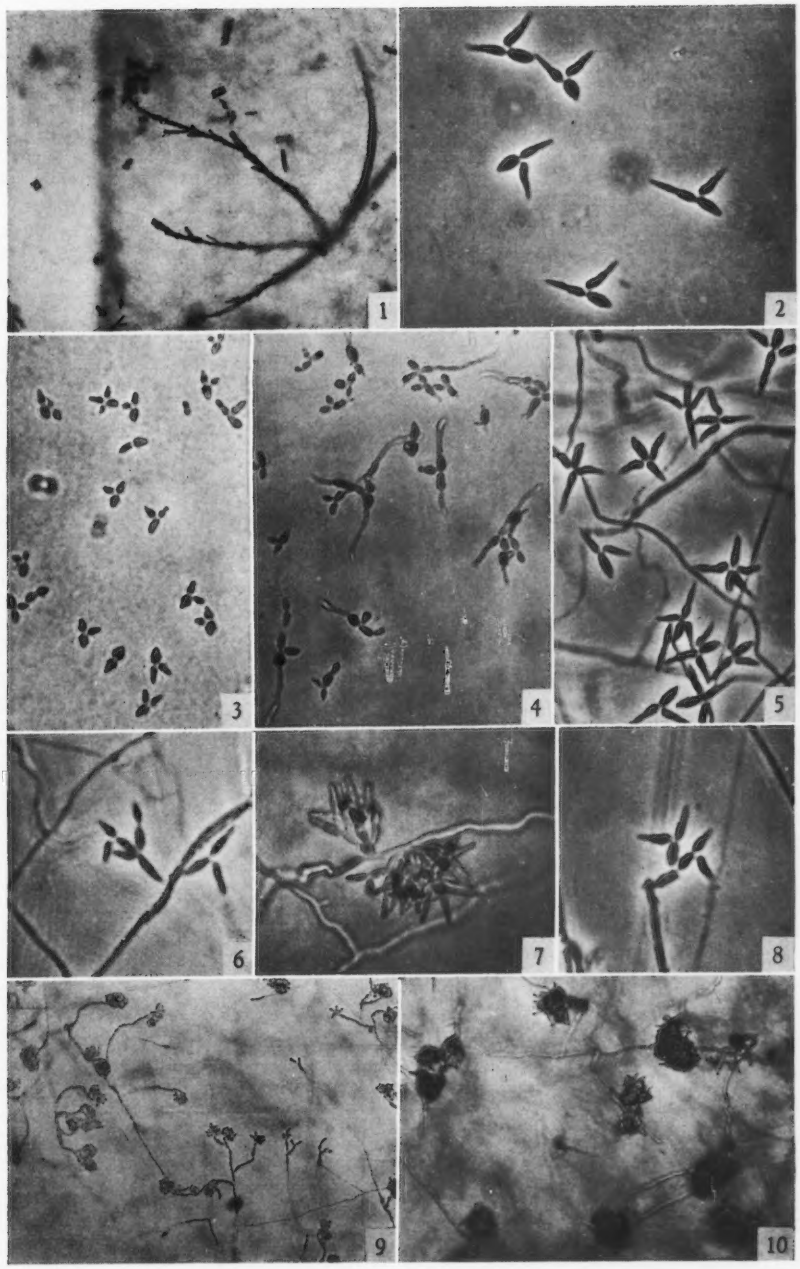
FIG. 7. Conidiophores with spores on surface agar plate culture. Phase × 666

FIG. 8. Conidiophore with two spores, lactophenol – cotton blue mount. Phase × 666

FIG. 9. Young sporophores on surface of agar plate culture. × 100

FIG. 10. Groups of spores on surface of agar plate culture. × 166

PLATE I



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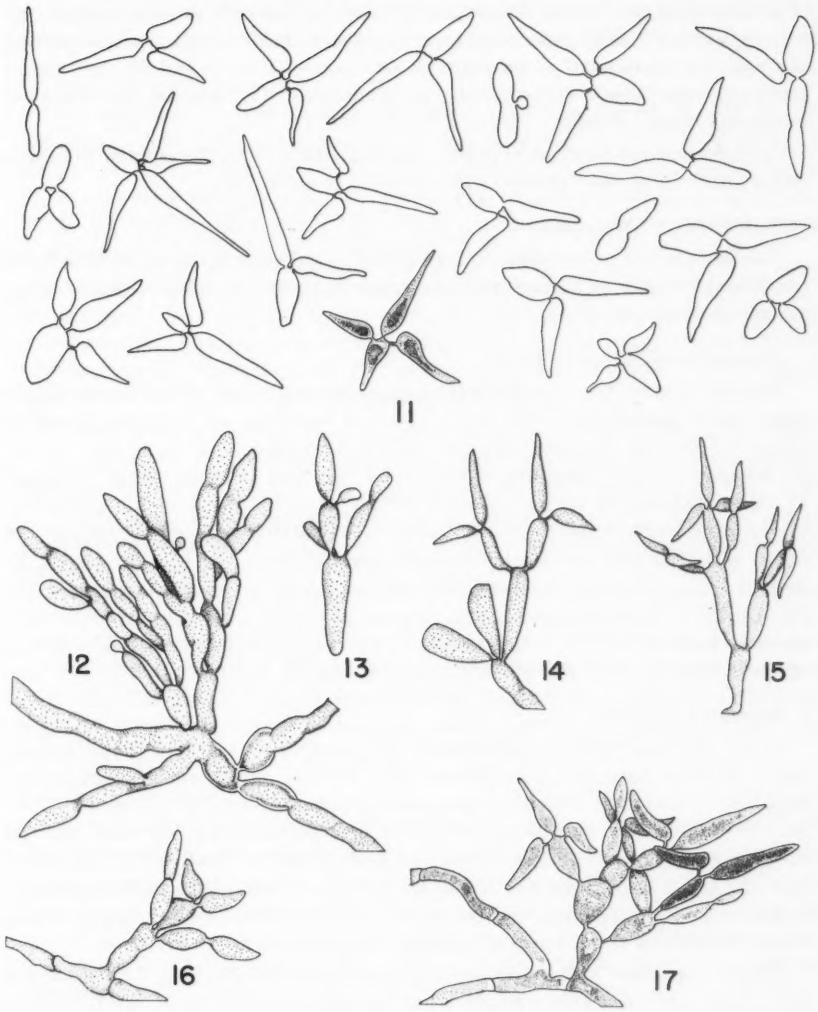


FIGS. 11 to 17. *Volucrispora aurantiaca.*
FIG. 11. Spores. Note variation in size, shape, and number of cells. × 1500
FIG. 12. Young compound sporophore on hypha from recently germinated spore. × 1000
FIG. 13. Young sporophore bearing spore with developing lateral cell. × 1000
FIG. 14. Sporophore bearing two nearly mature spores. × 1000
FIG. 15. Sporophore with spores in several stages of development. × 1000
FIG. 16. Young sporophore with developing spores. × 1000
FIG. 17. Sporophore with developing and mature spores. × 1000
All drawings were made with the aid of a projection apparatus.

followed by the apical and then the lateral cells (Fig. 4), though any sequence of germination may occur where one or more of the cells is abnormal in one way or another. The germ tube leaves the spore cell through a wide opening and may be constricted at its point of emergence (Figs. 4 and 12) but thereafter averages about two microns in diameter. The mature mycelium is somewhat more slender.

Germination has been observed to take place only from the ends of the spore cells remote from their points of attachment to each other.

Formation of Mycelium

The sequence of events that follows germination varies greatly with cultural conditions. A mat of surface and subsurface, septate, branched, anastomosing mycelium soon develops.

Formation of Conidiophores

The development of conidiophores and spores may start almost immediately after spore germination (Fig. 12). This is repeated at irregular intervals along the various hyphae. Spore formation occurs at or near the surface of the agar or liquid medium in which the fungus is growing (Figs. 7, 9, and 10). A single spore may be borne laterally on a hypha (Fig. 6, right side), or the conidiophore may be very simple, consisting of a single cell on the distal end of which several spores are borne (Figs. 6, 8, 13, and 16) or be several times or profusely branched before spores are formed (Figs. 7, 12, 15, and 17). The older parts of an agar colony surface are usually completely covered with conidiophores and their tangled masses of spores. The spores are produced in slime which may be very limited in amount.

Formation of Spores

It has not been determined whether or not spores may be formed successively from a terminal sporophore cell. When conditions of growth are suitable the terminal cells of a sporophore branch develop into a spore. The penultimate cell becomes the basal cell of the spore and the terminal cell, the apical cell. The lateral cells develop as buds from the basal cell of the spore, near the point of attachment of the apical cell. Usually the bud is spherical and attached to the basal cell by means of a narrow isthmus, though sometimes, depending on cultural conditions, the isthmus is broad.

The young lateral cell soon takes on an elongated shape (Fig. 13), enlarging and taking up a position reflexed from the apical cell (Figs. 2, 5, 11, etc.) in the mature spore. Spores may become detached before any lateral cell is formed. Spores bearing one lateral cell (Figs. 2, 6, and 8) or two lateral cells (Fig. 5) are most common. Three or more lateral cells on a spore are sometimes seen (Figs. 5 and 11).

The spore cell contents are darkly staining with a dense (under phase microscopic observation) nucleus-like mass taking up most of the intracellular space. The apical cell, and sometimes the lateral cells of a spore, are often somewhat centrally constricted with this nucleus-like mass present on

each side of the constriction. Where the apical and lateral cells are able to complete a cell division, the incipient spore appears not to be detached but remains on the sporophore to function as another branching of the sporophore.

Colony Characteristics

The colonies on Difco potato dextrose agar (incubated at 25°–28° C.) are smooth and flat but become raised and wrinkled at the points of inoculation and in the older parts of mature colonies. The margin is entire except in older drying colonies. Aerial hyphae are almost entirely lacking, limited to the occasional white tuft. While some colonies are not particularly moist in appearance, they are never powdery.

The color of the colony varies with the medium and with the method of inoculation. It is usually a pale orange-brown with the reverse side concolorous. On Difco potato dextrose agar the colony soon becomes a rich orange in color darkening somewhat as the red-brown metabolic product crystallizes out in and amongst the mycelial threads.

Neither coloration nor rate of growth are noticeably affected by light. Cultures grown in complete darkness, normal daylight, and in continuous light showed no noticeable color or growth rate differences. At 28° C. on Difco potato dextrose agar, a colony increases in diameter from a 1 mm.-wide piece of inoculum to 16 mm. in 3 weeks (Fig. 36). The fungus is relatively slow growing.

Habitat

The natural habitat of the fungus is not known. The present strain originated either from a soil sample or from an air contaminant during plating of a soil sample. In this geographical area where dust may be blown frequently and from long distances, a clear distinction between soil-borne and air-borne organisms is not always possible.

Taxonomic Position

Following the classification of the Hyphomycetes of Wakefield and Bisby (7) this fungus fits best with the subfamily of Staurosporae of the Gloiosporae. Its spore form reminds one of the spores of some of the aquatic Hyphomycetes so excellently described by Ingold (3). However, when the present fungus is grown in water its spores are produced at or near the surface and are released onto the surface film. Thus it is not considered to be an aquatic Hyphomycete.

The resemblance of the present fungus to *Titaea ornithomorpha* Trotter is discussed below. There is also some resemblance between our organism and *Triglyphium album*. However, the method of formation of the spores characteristic of the genus *Triglyphium* Fresenius (1) precludes the inclusion of our fungus in that genus.

Recently van Beverwijk (6) has described an organism *Tricellula inaequalis* as the type of the genus *Tricellula*, a genus, characterized, in part, by having conidia of three cells connected by narrow constrictions.

The spores of the present fungus average twice the size of the spores of *Tricellula inaequalis*. They are made up of from two to six cells (usually three to four) rather than the three of *T. inaequalis*. The cells are connected by a definite narrow isthmus rather than a constriction. The spores have been observed to germinate only from the ends of the cells remote from their points of attachment to each other. Conidiophores have not been observed on a germinating spore cell in addition to a germ tube as figured by van Beverwijk for *T. inaequalis*.

In view of these differences it is considered more advisable to make the present fungus the type of a new genus rather than to emend van Beverwijk's genus *Tricellula* to include an organism with characteristically three- to four-celled spores with cells of different shape and orientation. Because of the remarkable resemblance of the spores to birds in flight, the generic name *Volucrispora* is suggested with the present fungus as the type species.

Diagnoses

Volucrispora n. gen.

Fungus with branched anastomosing septate mycelium. Coniodiphores simple or compound. Conidium, produced in slime, consisting of a basal cell bearing at or near its distal end one to five cells. The one to five cells are connected to the basal cell by narrow isthmuses or constrictions.

Volucrispora gen. nov. (Lat. volucer—flying, winged, from the appearance and cell arrangement of the spore)

Mycelium ramosum septatum anastomosans. Conidiophora simplicia aut composita. Conidia (gloiospora) singula constantia e cellula basali ferente cellulas 1–5 e latere remoto. Cellulae 1–5 coniunctae sunt cum cellula basali per isthmos angustos vel iuncturas artas.

V. ***aurantiaca*** n. sp.

Fungus with branched, anastomosing septate mycelium. Conidiophore simple or compound. Conidium, produced in slime, of two to six cells, usually three to four. The basal cell 4–6 μ long by 1–3 μ wide, bearing on its often larger distal end a pointed apical cell 6–14 μ long, and, from near the point of attachment of the apical cell, none to four lateral cells 3–10 μ long. The apical and lateral cells connected to the basal cells by narrow isthmuses or constrictions.

Natural habitat unknown, isolated from a water culture containing soil collected near Saskatoon, Saskatchewan, Canada, February 3, 1956.

Cultures deposited with the American Type Culture Collection, the Centraalbureau voor Schimmelcultures, and the National Collection of Fungus Cultures, Kew, Surrey. Dried cultures deposited with the Herbarium, C.M.I., Kew, England, and the Farlow Herbarium, Harvard University, Cambridge, Mass.

V. ***aurantiaca*** sp. nov.

Mycelium ramosum septatum anastomosans. Conidiophora simplicia aut composita. Conidia liberata in limo, constantia e 2–6 cellulis plerumque

3–4. Cellula basalis 4–6×1–3 μ, ferens e latere remoto cellulam summam 6–14 μ longam; atque 0–4 cellulam obliquam, 3–10 μ longam. Cellula summa et cellulae obliquae coniunctae sunt cum cellula basali per isthmos angustos vel iuncturas artas.

Hab. ignota, collecta ex cultura in aqua, Saskatonia, Saskatchewanensis, Canada.

Volucrispora ornithomorpha (Trotter) n. comb.

The fungus, *V. aurantiaca*, resembles an organism named *Titaea ornithomorpha* by Trotter (5) as far as can be determined from Trotter's very brief specific description and outline figure of a single spore. The spore figured and described by Trotter, if inverted, is remarkably similar to the four-celled spores of our fungus (Figs. 5, 11, 15, and 17) and the specific name is particularly apt as duck-hunting mycologists will agree (see Fig. 2)! *V. aurantiaca* has not yet been isolated from, or successfully inoculated into, living leaves of *Cerastium* sp. as was Trotter's organism, though attempts are being continued.

As Ingold (3) has pointed out, "a comparison of the shapes of the spore in the several species of *Titaea* does not seem to suggest that these organisms fall naturally into a single genus and, if these fungi are rediscovered and investigated further, the genus may well have to be split". *Titaea maxilliformis* has already been removed from *Titaea* and has been named *Tetracladium maxilliformis* (Rostr.) Ingold (3).

If *Titaea ornithomorpha* can be considered to be the same genus as the present fungus, then the spore form and type of development differ sufficiently from the type species *Titaea callispora* Sacc. (4) to warrant the removal of *T. ornithomorpha* to another genus. In spite of the brevity of Trotter's description of *T. ornithomorpha*, it is considered that there is sufficient information to warrant the placing of his species in the same genus as the present organism, and its name, therefore, should become *V. ornithomorpha* (Trotter) n. comb.

Tricellula curvatis n. sp.

During the foregoing study, a lyophilized culture of *T. inaequalis*, originally isolated from the air in the vicinity of Atlanta, Georgia, U.S.A. (2) was received through the kindness of Dr. M. A. Gordon. Unfortunately this culture could not be revived from the lyophil state. A culture of Gordon's isolate was then purchased from the American Type Culture Collection (ATCC 11837, PRL 1561). The culture was propagated and the resulting cultures used in this study, which provides information additional to that published by Gordon (2).

Morphology and Development

The Spores

The spores of Gordon's Atlanta, Ga., isolate are 7–10 μ long, each consisting of three cells—one basal, one apical, and one lateral (Figs. 18 and 38). The basal cell, oblong to ovoid to triangular in shape and somewhat curved,

2–2.5 μ wide by 3–5 μ long, is attached to the sporophore at its smaller end (Figs. 28 and 39–41). On the larger distal end the apical cell, more or less triangular, pointed and curved, 2–2.5 μ wide by 3–5 μ long, is attached. The two cells are not in line with each other for the apical cell develops in a position to one side or at an angle with the longitudinal axis of the basal cell and retains this position at maturity (Figs. 27 and 39–41). The lateral cell develops as a spherical bud (Fig. 39), takes on a more or less triangular, pointed, curved shape and size similar to that of the apical cell, and assumes a position generally at right angles to the other two cells (Figs. 18, 38, and 40, 41). The apical and lateral cells are connected to the basal cell by means of a short, narrow but distinct isthmus. It is not known whether there is a crosswall at the isthmus or whether the passageway has merely been occluded by a thickening of the spore and isthmus walls. One- and two-celled spores have been observed (Figs. 20 and 38). These are probably immature or abnormal. The spores separate readily from sporophore, but the individual cells remain firmly together even when roughly handled. A spherical, nucleus-like body can be seen in each cell of the spore with suitable lactophenol – cotton blue staining and with phase microscopy (Figs. 18 and 38).

Germination of Spores

At germination, the spore swells up considerably, the pointed ends of the cells become rounded (Figs. 19–20), and one germ tube develops usually from each cell of the spore, from the end of the cell remote from its attachment to the other cell(s) of the spore (Figs. 20–23).

Formation of Mycelium

The rate of growth of *T. curvatis* is faster than that of *T. inaequalis* (see below) or of *V. aurantiaca* (Figs. 36, 37). A 1-mm. piece of inoculum developed into a colony 29.5 mm. in diameter when grown on Difco potato dextrose agar at 28° C. for 3 weeks (Fig. 36).

FIGS. 18 to 28. *Tricellula curvatis.*

FIG. 18. Spores from surface of agar culture. Lactophenol – cotton blue mount. Phase × 666

FIG. 19. Spores on cellophane on agar surface after 12 hours at 28° C. Individual living cells have swollen considerably. Moribund cells remained unchanged. Phase × 333

FIG. 20. Germinating spores, on agar surface, after 16 hours at 28° C. Phase × 333

FIG. 21. Germinating spores, on agar surface, after 16 hours at 28° C. Phase × 333

FIG. 22. Germinating spores, on cellophane on agar surface, after 16 hours at 28° C. Phase × 333

FIG. 23. Young fungus plants, on cellophane on agar surface, after 24 hours at 28° C. Phase × 333

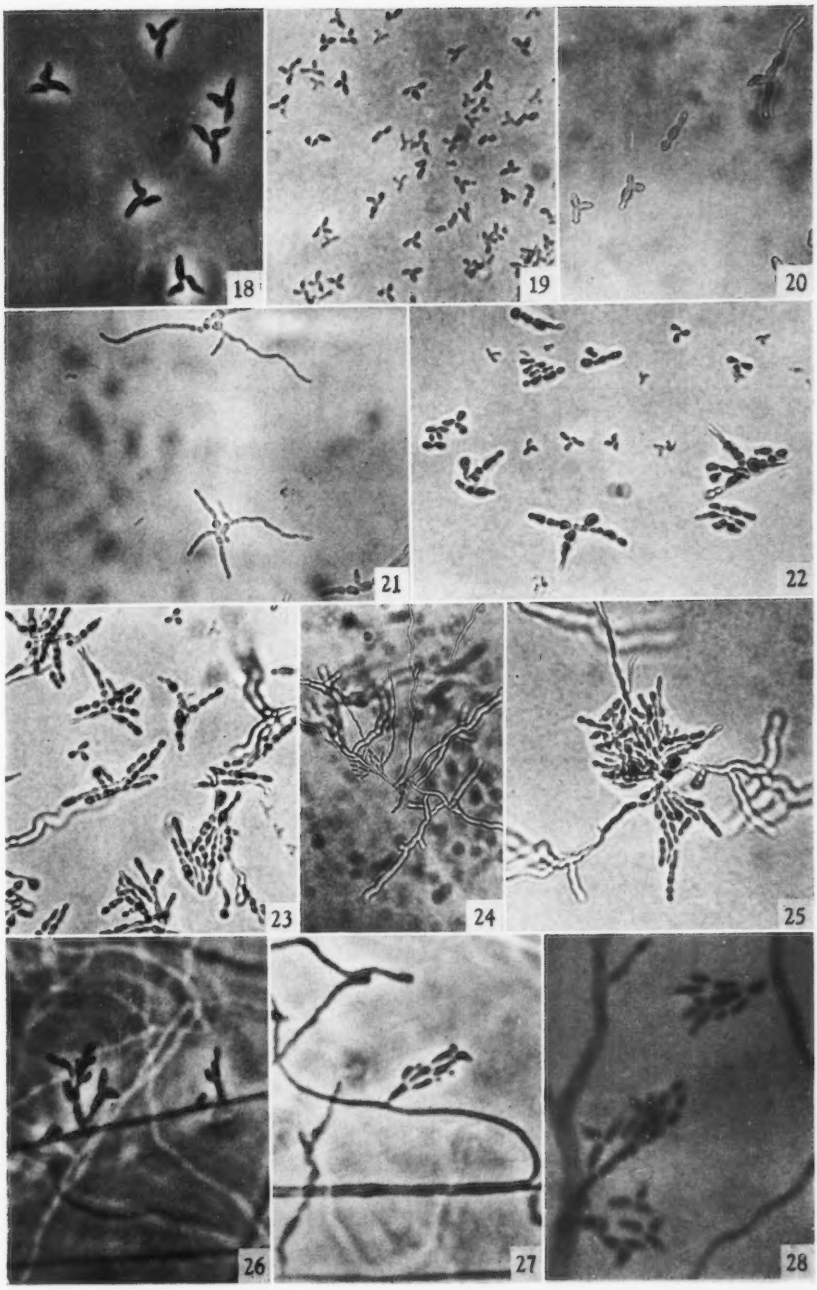
FIG. 24. Young fungus plant showing compound sporophore developing on hypha near remnants of germinated spore, on agar surface, after 36 hours at 28° C. × 333

FIG. 25. Compound sporophore developing near remnants of germinated spore, on cellophane on agar surface, after 40 hours at 28° C. Phase × 333

FIG. 26. Young sporophores on mature mycelium. Note apical cell developing out of line with basal cell. Lactophenol – cotton blue mount. Phase × 666

FIG. 27. Young sporophore on mature mycelium. Note apical cells developing out of line with basal cell. Lactophenol – cotton blue mount. Phase × 666

FIG. 28. Sporophores bearing mature spores. Lactophenol – cotton blue mount. Phase × 666



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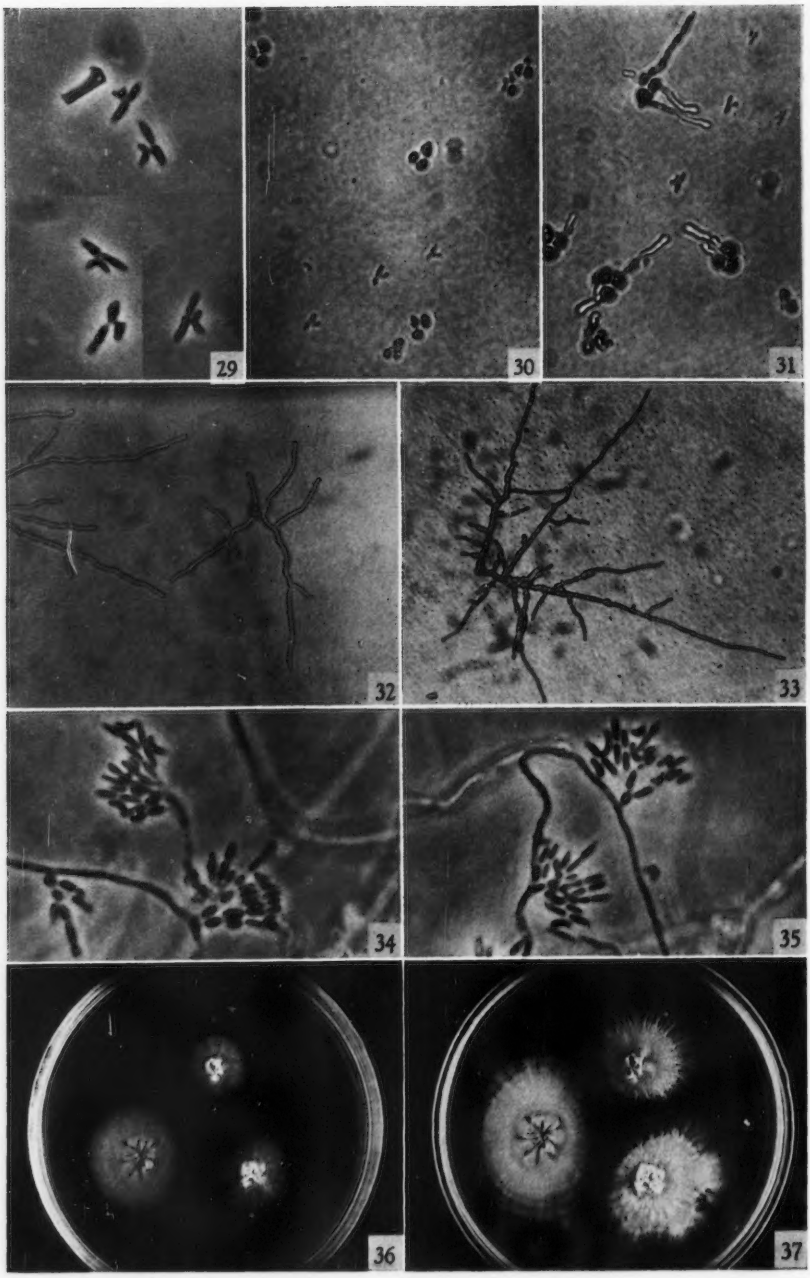

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Formation of Conidiophores

Sporophore formation may begin soon after germination (Figs. 24 and 25) and thereafter may occur anywhere along the branching septate mycelium close to or at the surface of the medium in which it is growing (Figs. 26–28).

Formation of Spores

While three cells per spore has been the maximum number observed during examination of many cultures and thousands of spores, the fact that the sporophore is occasionally more than dichotomously branched indicates that perhaps four-celled spores could occur.

Colony Characteristics

The colony is smooth, flat, rather dry (never powdery) with inverted folds radiating from the older raised portions of the colony (Figs. 36, 37). Its color is dull white to cream on Difco potato dextrose agar contrasting with the light orange of *T. inaequalis* and the darker orange-brown of *V. aurantiaca*.

Habitat

The fungus was first isolated from the air in the vicinity of Atlanta, Ga. (2), therefore the natural habitat is unknown.

Taxonomic Position

Gordon's Atlanta, Ga., isolate (PRL 1561) is obviously very closely related to *T. inaequalis*, but its characteristic spore shape, its rate of growth, and its colony characteristics all differ from those of *T. inaequalis*. These differences are considered sufficient to warrant naming the Atlanta isolate a new species of *Tricellula*. To do this, the generic description of *Tricellula* must first be emended.

FIGS. 29 to 35. *Tricellula inaequalis*.

FIG. 29. Spores from surface of agar culture. Lactophenol – cotton blue mount. Phase × 666

FIG. 30. Spores on cellophane on agar surface after 12 hours at 28° C. Living cells have swollen characteristically, moribund cells remain unchanged. Phase × 333

FIG. 31. Germinating spores on cellophane on agar surface after 16 hours at 28° C. Phase × 333

FIG. 32. Young fungus plant on cellophane on agar surface after 24 hours at 28° C. Phase × 200

FIG. 33. Developing fungus plant on cellophane on agar surface after 36 hours at 28° C. Phase × 200

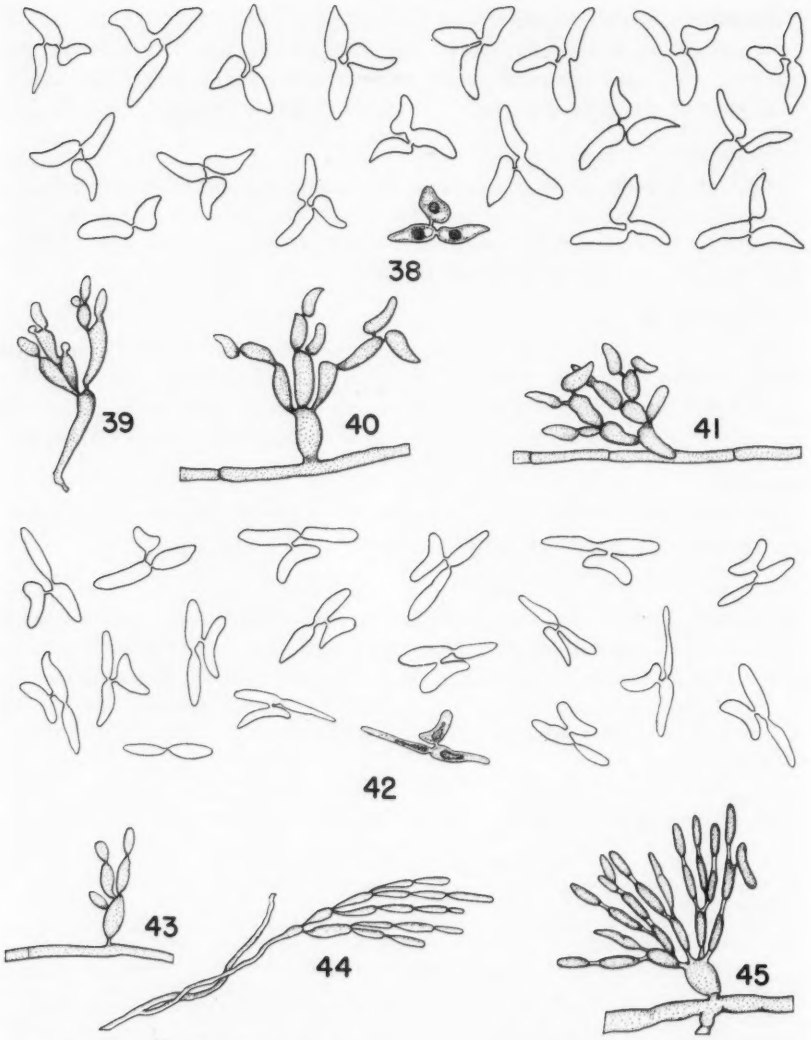
Fig. 34. Sporophores on mature mycelium. Note mature spores, apical and basal cells in line at top of figure. Lactophenol – cotton blue mount. Phase × 666

FIG. 35. Sporophores on mature mycelium with spores in various stages of development. Lactophenol – cotton blue mount. Phase × 666

FIGS. 36 and 37. Rate of growth comparisons.

FIG. 36. Potato dextrose agar plate culture after 3 weeks at 28° C. Smallest colony is *V. aurantiaca*, largest colony is *T. curvatis*, the other colony is *T. inaequalis*. × ½ approximately.

FIG. 37. Same plate as Fig. 36, after 5½ weeks at 28° C. Margins of colonies are typical of those on drying agar in crowded cultures. × ½ approximately.



FIGS. 38 to 41. *Tricellula curvatis.*
FIG. 38. Spores, showing variation in size and shape. × 1500
FIG. 39. Sporophore, showing lateral cell developing on young spores. × 1000
FIG. 40. Sporophore. Note apical cell of spores out of line with longitudinal axis of basal cell. × 1000
FIG. 41. Sporophore with nearly mature spores. × 1000
FIGS. 42 to 45. *Tricellula inaequalis.*
FIG. 42. Spores, showing variation in size and shape. × 1500
FIG. 43. Young sporophore. × 1000
FIG. 44. Terminal, compound, dichotomously branched sporophore. × 1000
FIG. 45. Short compound sporophore. Note apical cell of spores in line with basal cell. × 1000
All drawings were made with the aid of a projection apparatus.

Diagnoses

Tricellula van Beverwijk 1954 emend.

Fungus with branched, septate mycelium. Conidiophore simple or compound. Conidium (phialospore, gloiospore) three-celled, one cell basal, one cell apical, and the third lateral. The apical and lateral cells connected to the distal end of the basal cell by narrow isthmuses or constrictions.

Tricellula van Beverwijk 1954 emend.

Mycelium ramosum, septatum. Conidiophora simplicia aut composita. Conidia (phialosporae, gloiosporae) singula constantia e ternis cellulis, cellula prima basalis, cellula secunda summa, cellula tertia obliqua. Cellula summa atque cellula obliqua coniunctae sunt cum latere remoto cellulae basalis per isthmos angustos vel iuncturas artas.

Because the cells of the spore of the Atlanta, Ga., isolate (PRL 1561) are generally characteristically curved and the apical cell is at an angle and not in line with the longitudinal axis of the basal cell, the name *Tricellula curvatis* is suggested.

T. ***curvatis*** n. sp.

Fungus with branched, septate, mycelium, conidiophore simple or compound producing groups of phialides. Conidium 2–7 μ wide by 7–10 μ long of three cells, each cell measuring about 2–2.5 μ wide by 3–6 μ long. Basal cell oval to triangular shaped. Apical and lateral cells more or less triangular with somewhat pointed free ends, and connected to the basal cell at or near its distal end by narrow isthmuses or constrictions. Longitudinal axis of apical cell not in line with that of basal cell. Spores liberated in slime.

Natural habitat unknown, collected from air, near Atlanta, Georgia, U.S.A., October 28, 1953, by M. A. Gordon.

Cultures deposited with the National Collection of Fungus Cultures, Kew, Surrey. Dried cultures deposited with the Herbarium, C.M.I., Kew, England, and the Farlow Herbarium, Harvard University, Cambridge, Mass.

T. ***curvatis*** sp. nov.

Mycelium ramosum, septatum. Conidiophora simplicia aut composita, formantia phialidas conglobatas. Conidia 2–7×7–10 μ, singula e ternis cellulis constantia; unaquaeque cellula 2–2.5×3–6 μ. Cellula basalis ovata sive triangula. Cellulae summae et obliquae aliquo modo triangulae coniunctae sunt cum cellula basali e latere remoto per isthmos angustos vel iuncturas artas. Conidia liberata in limo.

Hab. ignota, collecta ex aëre, prope Atlantam, Georgiae, U.S.A.

Tricellula inaequalis van Beverwijk

The studies on this species, reported herein, are based on observations made on cultures (PRL 1563) propagated from a culture of *T. inaequalis* van Beverwijk (Bergen Norway isolate) purchased from the Centraal Bureau voor Schimmelcultures in 1956.

The isolate agrees very closely with Miss van Beverwijk's description (6) of this fungus. The few minor differences observed are described here and illustrated in the accompanying figures.

Morphology and Development

The Spores

The spore of *T. inaequalis* differs from that of *T. curvatis* in that the longitudinal axis of the apical cell is characteristically in line with that of the basal cell and the lateral cell is attached sidewise to the basal cell of the spore (Figs. 29, 34, 42, and 45). The three cells are connected, usually, by a distinct narrow isthmus (Fig. 29, etc.), often merely by a narrow isthmus. It is not known if there is a crosswall between the cells or if the opening is merely occluded by thickening of the spore wall.

In drying cultures many variations of spore shape are observed. At maturity in moist cultures only the lateral cell tends to be triangular or boat-shaped, the other two cells becoming more or less oblong.

Germination of Spores

At germination the cells of the spore swell up considerably into more or less spherical bodies (Figs. 30–33) and present an aspect quite distinct from that of germinating spores of either *V. aurantiaca* (Figs. 3, 4) or *T. curvatis* (Figs. 19–21). No more than one germ tube has been observed to develop from any one of the cells of the spore. Any or all of the cells may produce germ tubes.

Formation of Mycelium

The rate of growth of the fungus is slower than that of *T. curvatis* (Figs. 36, 37). A 1-mm. piece of inoculum developed, on Difco potato dextrose agar at 28° C., into a colony 21 mm. in diameter in 3 weeks (Fig. 36).

Formation of Conidiophores

Sporophores have not yet been seen to develop directly from an already germinating spore cell, as figured by van Beverwijk (6). Sporophores have been observed to develop on the mycelium immediately after germination and thereafter occur at irregular intervals at or near the surface of the medium, along the branching septate mycelium (Figs. 34, 35).

Probably because of a more regular dichotomous branching and the fact that the cell contents are densely staining while the constricted areas between the cells stain lightly, the sporophore of *T. inaequalis* in our cultures (Figs. 34, 35) look characteristically different from the sporophores of *T. curvatis* (Figs. 26–28).

Three cells per spore was the maximum observed. The sporophores are usually dichotomously branched (Fig. 44) though very occasionally more than dichotomy has been observed (Figs. 43 and 45).

Formation of Spores

The lateral cell of the spore develops in much the same way that the lateral cell of *T. curvatis* (see above) develops. However, as the spore approaches

maturity, the lateral cell assumes its sidewise position to the basal cell (Figs. 29, 34, and 35) with the isthmus connecting it to the basal cell located often nearly midway along its convex side.

Colony Characteristics

The colony is smooth, moist or somewhat dry but never powdery, becoming raised at points of inoculation. Aerial hyphae are limited to sparse white tufts. The color, on Difco potato dextrose agar, becomes a pinkish orange.

Acknowledgments

The author is indebted to Mr. R. F. Cole of this laboratory for preparation of the photographic prints from negatives provided, and to Dr. Pieter de Jong, St. Andrews College, Saskatoon, Saskatchewan, for translation of the diagnoses into Latin.

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MANOMETRIC GAS ANALYSIS AS AN INDEX OF MICROBIAL OXIDATIONS AND REDUCTIONS IN SOIL[1]

C. M. GILMOUR, L. DAMSKY, AND W. B. BOLLEN

Abstract

A manometric procedure for the determination of microbial oxidations and reductions in soil is described. The results indicate that over-all microbial activity in treated and non-treated test soils may be accurately evaluated by oxygen consumption values and oxygen depletion points. It is also shown that active gas loss via denitrification may be detected manometrically by measurement of gas evolution following oxygen depletion.

Microbial respiration as observed by carbon dioxide evolution has assumed an important role in the study of the level of biochemical activity of the soil microflora. This experimental approach has been extended to include free oxygen used in the oxidation of carbon and to the liberation of gases other than carbon dioxide. All are involved in the terminal respiratory activity of particular soil organisms.

Recently, Bunt and Rovira (1), Chase and Gray (2), Gilmour *et al.* (3), and Katznelson and others (4) provided reports on the use of the Warburg respirometer to study microbial metabolism in soils. These workers were of the opinion that the Warburg method for the determination of oxygen uptake gives an accurate measure of respiratory activity in soil. Our studies indicate that observation of gas consumption and evolution may be used not only in following the breakdown of organic materials in soil, in confirmation of previous reports (5), but also for the detection of soil nitrogen losses via denitrification.

Experimental

Soil Treatment

In preliminary experiments, several soils were tested. These included Chehalis silt loam and Brookston silt loam. However, Palouse silt loam soil from northeastern Oregon was used throughout the greater part of the study. After air-drying, each soil was passed through a 10-mesh screen and stored in airtight metal containers.

Fifty-gram portions of soil on a water-free basis were weighed out and placed in 125-ml. Warburg flasks. When desired, 60-mesh wheat straw was mixed with soil at a rate equivalent to 20 tons per acre. Soil moisture in each flask was adjusted to 50% of saturation; water-soluble chemicals such as glucose, $NaNO_3$, NH_4NO_3, and $(NH_4)_2SO_4$ were added in certain cases with the water. Glucose was added at a rate of 0.25 g. to 50.0 g. of soil, and the nitrogen fertilizers when added were in amounts to provide 0.050 g. of available nitrogen.

[1]Manuscript received February 11, 1958.
Contribution from the Department of Bacteriology, Oregon State College, Corvallis, Oregon. This investigation was supported in part by a grant from the Western Soil and Water Research Committee, Regional Project, W-31. Published as Technical Paper No. 1108 with the approval of the Director, Oregon Agricultural Experiment Station.

Respiration Procedure

Large Warburg flasks (125 ml.) having a center well, vented side-arm, and standard taper ground-glass neck, were used. This type of flask permits use of a much larger soil sample (50.0 g.) than would be the case with the standard 25.0-ml. Warburg flask. Two milliliters of 20% KOH was added to the center well and folded filter paper placed in each well so as to absorb any carbon dioxide evolved. Individual flasks were then attached to calibrated manometers and placed in a constant temperature water bath (28° C.). In the case of each soil treatment, appropriate non-treated controls were included. Since the latter reflect the normal gas exchange of the non-treated soil, the term "endogenous" respiration was applied. All oxygen consumption results are expressed in terms of microliters gas consumed. Since with gas evolution involving denitrification a number of gases may be evolved, only millimeter change in manometer level has been reported (Fig. 4).

Experimental Results

Non-treated Soils

In early experiments various non-treated soils were tested to obtain data relative to the extent of oxygen consumption. The results obtained with three different soils are depicted in Fig. 1. It is evident that considerable variation exists between the three soils in respect to oxygen taken up by the soil flora. Soil 1, Chehalis silt loam, consumed over 1500 μl. of oxygen during 10 hours' incubation; soil 2, Palouse silt loam, consumed approximately 1000 μl.; and soil 3, Brookston silt loam, only 400 μl. of oxygen. These data attest to the possible application of oxygen consumption values in the evaluation of general microbial activity in test soils.

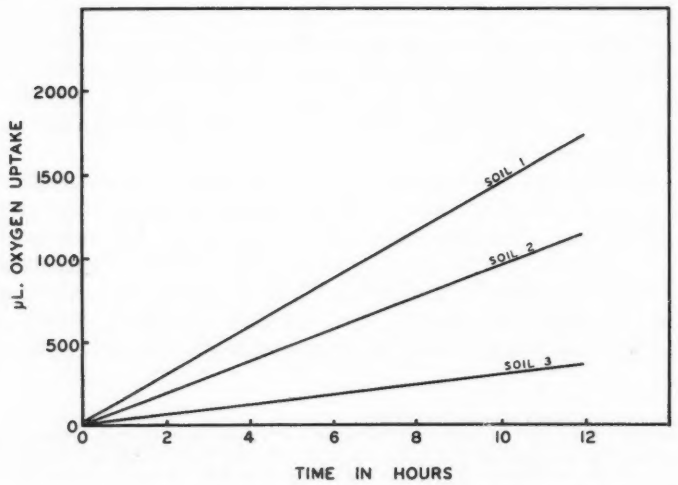

FIG. 1. Observed differences in oxygen uptake with three soil types.

Treated Soils

The inclusion of organic matter and inorganic nitrogen might be expected to alter the growth and metabolism of the soil flora. Reference to Fig. 2 discloses that the addition of glucose stimulated oxygen consumption 30 to 40 times over that observed in the untreated soil. The inclusion, however, of sodium nitrate brought about an apparent decrease in oxygen uptake when uptake was compared to the respiration values observed with glucose alone.

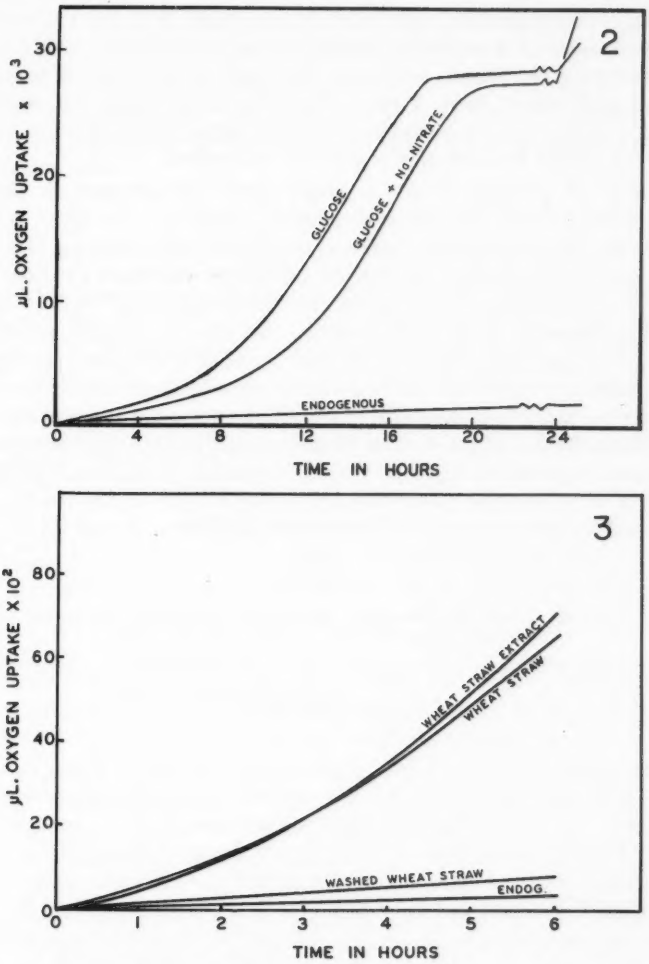

FIG. 2. Influence of glucose and nitrate nitrogen on oxygen consumption with Palouse silt loam soil.

FIG. 3. Comparative oxygen consumption values obtained with wheat straw and soluble components of the straw in Palouse silt loam soil.

The plateau shown in Fig. 2 starts at 18–20 hours and results from depletion of oxygen. At the point indicated by the broken line, air was reintroduced and normal uptake began immediately.

As in the case of glucose, wheat straw also causes an increase in oxygen consumption by the soil flora (Fig. 3). The stimulation of soil respiration by the inclusion of wheat straw is surprisingly rapid and of long duration. Presumably this effect could only be attributed to the water-soluble fraction of wheat straw or that fraction immediately available for dissimilation by the soil flora. In this regard, a study of Fig. 3 shows that little stimulation in respiratory activity was obtained with washed wheat straw and that wheat straw extract (cold water) produced the same level of stimulation as was obtained with wheat straw alone. This observation takes on added significance in respect to later comments on soil nitrogen losses in which the aforementioned soluble fraction of wheat straw is involved.

The over-all influence of added wheat straw and nitrogen on the rate of oxygen uptake in the test soil is depicted in Table I. The given respiration periods showed no evidence of oxygen depletion. The time periods between those listed in Table I (20–52 hours, 72–100 hours) were used to allow aeration of the Warburg flasks. It is evident that the inclusion of NO_3-N with wheat straw has brought about a decrease in the rate of oxygen uptake. This effect is more pronounced during the 52–72 and 100–120 hour periods. This finding takes on added significance should the observed drop in oxygen consumption be a reflection of the role of NO_3-N as a terminal acceptor of hydrogen or of the production of gases other than carbon dioxide. Either effect might be expected to cause an apparent drop in oxygen consumption. Further support for a rather specific respiration role of the NO_3-N is given in the stimulation of soil oxygen uptake with ammonium sulphate. In the latter instance,

TABLE I

OBSERVED INFLUENCE OF WHEAT STRAW AND NITROGEN FERTILIZER ON OXYGEN CONSUMPTION IN PALOUSE SILT LOAM

Soil treatment	Respiration period (hours)					
	0–20		52–72		100–120	
	Q_{O_2}*	Q_{O_2}†	Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
Soil alone	1.7	0.8	1.8	0.8	1.9	0.9
Soil + wheat straw	26.5	12.4	16.3	7.8	12.6	5.9
Soil + wheat straw + $NaNO_3$	25.9	12.1	12.7	5.9	9.3	4.3
Soil + wheat straw + NH_4NO_3	25.3	11.8	8.5	3.1	9.1	4.2
Soil + wheat straw + $(NH_4)_2SO_4$	27.8	13.0	22.9	10.7	14.0	6.5

*μl./g. of soil/hour.
†μl./mg. of soil N/hour.

the stimulatory effect on microbial respiration was observed during all three time periods. It should also be mentioned that the gradual decrease in Q_{O_2} values over the entire time period (0–120 hours) reflects the decrease in supply of oxidizable carbon.

The afore-mentioned drop in oxygen consumption values noted in the presence of NO_3-N is of special interest since an identical effect has been observed with carbon dioxide evolution. Typical data are shown in Table II. With the level of NO_3-N used, the depression in carbon dioxide evolution is evident at a very early stage and persists for 24–40 hours. With higher concentrations of NO_3-N, little or no carbon dioxide is evolved in the first 24-hour period. As with all NO_3-N additions, however, the lag in carbon dioxide evolution disappears after 24–48 hours. Thus total carbon dioxide evolved after 135 hours' incubation is essentially the same in the presence or absence of NO_3-N.

TABLE II

OBSERVED EFFECT OF NITROGEN ON CUMULATIVE EVOLUTION OF CARBON DIOXIDE FROM PALOUSE SILT LOAM

Soil treatment*	mg. C as CO_2 evolved (hours)				
	12	20	43	85	135
Soil alone	1.52	2.40	3.92	5.57	7.04
Soil + glucose	10.30	27.70	42.70	54.95	59.85
Soil + glucose + $NaNO_3$	3.05	10.10	42.10	53.80	58.57
Soil + glucose + NH_4NO_3	5.17	24.89	41.54	52.09	55.99
Soil + glucose + $(NH_4)_2SO_4$	9.02	28.74	46.34	54.16	58.01

*Glucose and nitrogen additions as given under soil treatment.

The apparent decrease in oxygen consumption and carbon dioxide evolution in the presence of free nitrate parallels the production of gases other than carbon dioxide. Such might be predicted under conditions of active denitrification and presumably may be detected manometrically by the measurement of any increase in gas pressure within the closed flask system. Thus in contrast to prior runs, air was not readmitted to the flasks after depletion; carbon dioxide was absorbed in potassium hydroxide. Reference to Fig. 4 discloses that once air becomes limiting (20–24 hours), marked increases in gas pressure were noted whenever NO_3-N was included with wheat straw. No evidence of gas evolution was observed in the absence of wheat straw. Since mass spectrometer analysis of similar flask gas mixtures showed no methane or hydrogen evolved, it appears highly probable that nitrogen and/or the oxides of nitrogen stand as the principal gases evolved.

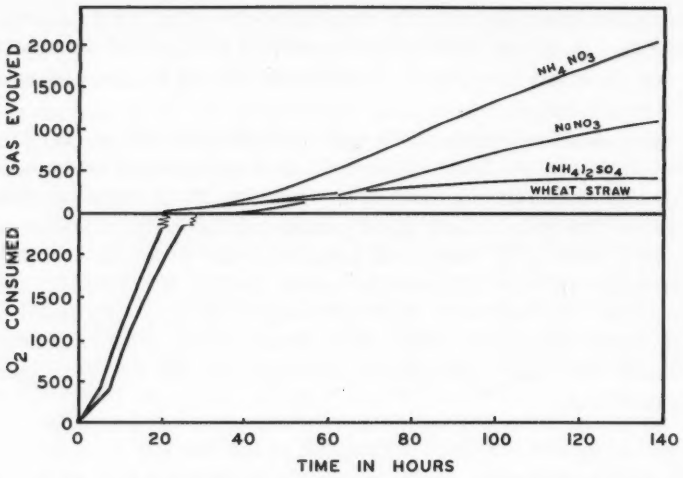

FIG. 4. Observed point of oxygen depletion and evolution of gas in Palouse silt loam soil treated with wheat straw alone and wheat straw plus inorganic nitrogen.

Discussion

The detection and measurement of gases consumed and evolved by the soil microflora has particular value when carbon utilization is being studied. Oxygen consumption values provide an index of the level of oxidizable carbon in the soil. In this regard, it should be emphasized that only carbon entering the cells undergoes oxidation. On the other hand, the more complex polysaccharides, proteins, and fats may be detected only when significant levels of the oxidizable (water-soluble) components of such compounds have accumulated in the soil. This problem may be partially overcome by adequate preincubation of soil samples prior to testing with the Warburg respirometer. In most instances a significant accumulation of oxidizable carbon then occurs which may be evaluated by measurement of the oxygen uptake values. In the natural soil system, however, a considerable amount of carbon is directly incorporated into cell substance and thereby does not undergo complete oxidation.

In classical manometric work involving isolated enzyme systems, pure cultures, and a defined synthetic substrate, the gas consumed is oxygen and the gas evolved carbon dioxide. Thus the interval and total manometer changes and subsequent calculations for the volume of gas consumed and evolved are usually based on the premise that gases other than oxygen and carbon dioxide are not involved. This undoubtedly is the case in studies of relatively short time periods (2–3 hours). In experiments of long duration, however, there is the possibility of gases such as hydrogen, methane, nitrous oxide, and nitrogen exerting a direct effect on the pressure of the closed system. Depending on the gas volumes involved, a small to rather large error in oxygen

uptake and carbon dioxide evolution values would result. It is suggested, therefore, that with soils containing high levels of soluble carbon, manometric runs be checked for the presence of interfering gases. As was shown in the present investigation, gases other than carbon dioxide are evolved as soon as oxygen becomes limiting.

The reduction in oxygen uptake and carbon dioxide evolution in the presence of nitrate and oxidizable carbon might be regarded as presumptive evidence for the role of nitrate as a terminal acceptor of hydrogen. On the other hand, stimulation of microbial growth in the presence of added nitrogen and carbon and subsequent rise in cell endogenous carbon might effect a net delay in oxygen uptake and carbon dioxide liberation. In the absence of added nitrogen, all terminal respiratory activity may be increased and thereby result in a greater percentage of the available carbon being oxidized to completion. Whatever the case, the afore-mentioned ideas attest to the specific influence of the nitrate radical and soluble carbon level in the gas exchange picture in soil.

At low levels of free oxygen, there seems little question that under the described experimental conditions, the manometric method supplies data relative to the evolution of gases from soil. It appears likely, therefore, that this procedure provides a rapid means for studying anaerobic denitrification and with further refinement should lend itself well to studies of the factors influencing denitrification.

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NUCLEAR STREAMING IN GELASINOSPORA[1]

E. SILVER DOWDING[2]

Abstract

Under some conditions, large numbers of nuclei of *Gelasinospora tetrasperma* travel through the mycelium for long distances, passing from one cell into the next via the septal pore. They are carried by the streaming cytoplasm at speeds as high as 40 mm. per hour. Under other conditions, the moving cytoplasm leaves all the nuclei behind, fixed in the thin cytoplasmic lining of the cell wall. Nuclei have been observed to stream in certain mated homokaryons but not in unmated ones. A mechanism for nuclear streaming is suggested.

By contrast-phase microscopy the migration of living fungal nuclei has been observed in the Ascomycetes and in the Basidiomycetes. Dowding and Bakerspigel (4) in *Gelasinospora tetrasperma* saw nuclei travel slowly through the septal pore from one cell into the next. Girbardt (7) in *Polystictus versicolor* saw conjugate pairs of nuclei migrate at the time that clamp connections were being formed in the hyphae. The speed of migration of the *Polystictus* nuclei was approximately equal to that of hyphal elongation. Such slow speed does not account for the following observations, which indicate much more extensive and rapid nuclear migration.

In *Coprinus lagopus* a haploid mycelium 5.6 cm. in diameter can be converted into the diploid state by the nuclei from an adjacent inoculum of opposite mating type within 3 or 4 days (2). Buller calculated that the average speed of the nuclei travelling from the inoculum through the large mycelium must have averaged 1.5 mm. per hour, about 10 times the rate of elongation of the hyphae. The number of nuclei required to diploidize such a mycelium must have been of the order of 250,000.

Similarly in *G. tetrasperma*, in order to produce heterokaryotic transformation, many thousands of nuclei must travel from one homokaryotic mycelium into another at a rate of at least 4.5 mm. per hour, that is, two or three times the hyphal growth rate.

Such a rapid migration of many nuclei, something which was previously only postulated, has now been actually observed and is recorded here. To distinguish this movement from slower nuclear movements, it is called "nuclear streaming".

Material and Methods

Selections of Suitable Strains

To observe nuclear migration one must use a pair of homokaryons that together fruit vigorously. Strains of *G. tetrasperma* differ widely in their fertility; not only do the heterokaryotic strains derived from normal-sized

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Contribution from the Department of Bacteriology, University of Alberta, Edmonton, Alberta, made possible by a grant to Dr. R. D. Stuart from the National Research Council, Ottawa, Canada.
[2]Mrs. E. S. Keeping.

ascospores vary, but also mated homokaryons derived from dwarf ascospores. Among appropriately mated homokaryons, the most fertile are those between mutant and wild strains (such as mutant 4 and wild 10) and these, to judge by the perithecia, show the most extensive nuclear migration (5). The perithecia appear much earlier than in other cultures, are very dense, and occur not only along the line where the two mycelia meet but all over the side occupied by the wild strain.

In microcultures designed to show nuclear streaming, strains 4 (mutant) and 10 (wild) were used side by side. Stock cultures of these strains were never more than 3 days old when they were subcultured, so that they were growing very rapidly when they were inoculated on to the microcultures.

Technique of Manipulation

To prepare a microculture as shown in Fig. 1, a drop of melted yeast-enriched malt agar is placed on the cover slip (*a*), inoculated, turned over, placed upon the slide (*b*), and pressed down to spread the agar. The agar should cover half the area between the slide and cover slip, and not extend to the margins. The culture is incubated in the dark in the Petri-dish damp chamber (*c*) at 30–33° C. until a fringe of stiff aerial hyphae (*d*) has grown beyond the cover slip. This takes one or more days depending on the amount of agar and the temperature. A culture is judged to be of suitable age if hyphae have fused and rudimentary fruit bodies have developed.

When the culture is placed upon the stage of the miscroscope, any movement of the cover slip and also any overexposure of the culture to dry air will damage the cells, and must therefore be avoided. A satisfactory culture contains no injured cells. Injured cells can be recognized because they are almost completely devoid of cytoplasm and are cut off from the adjacent cells of the hypha by bulging plugged septa (Fig. 4).

The space between the periphery of the agar and the edge of the cover slip must be kept filled with water by adding drops from time to time from a pipette to the slide. The preparation remains suitable for examination

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FIGS. 1 to 5. *Gelasinospora tetrasperma*, mutant × wild.

FIG. 1. A, B, and C, preparation of a microculture as described in text.

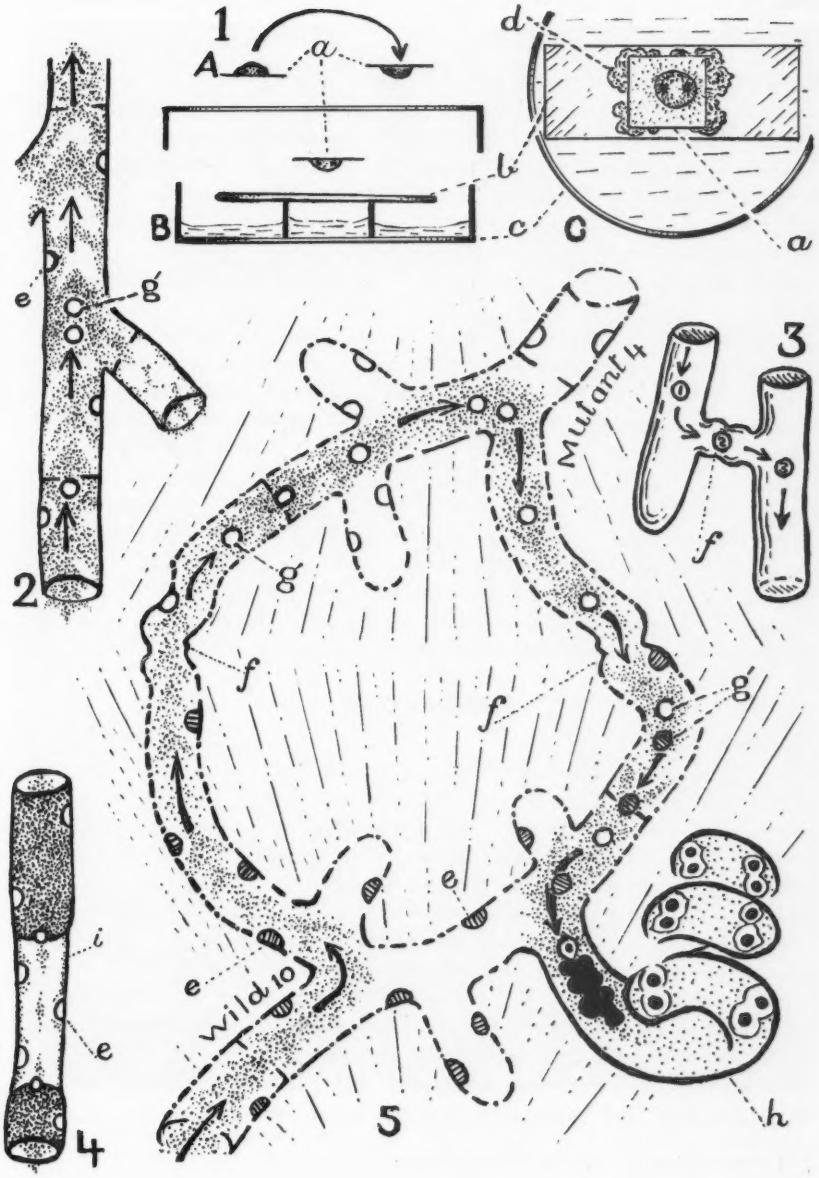
FIG. 2. Main hypha with cytoplasm streaming backwards from apex in the direction of the arrows, and with four stationary and three migrating nuclei.

FIG. 3. Three successive positions of a nucleus migrating out of one hypha into another via the peg-to-peg hyphal fusion.

FIG. 4. An injured cell cut off from the rest of the hypha by two bulging septa each with a plugged perforation. Most of the cytoplasm has been withdrawn leaving three nuclei and a lining layer of cytoplasm.

FIG. 5. Diagram of two fused homokaryons to show how one of them (10) might be converted to the heterokaryotic state. The radiating lines indicate the direction of growth of the two strains. The cytoplasm 10 enters the homokaryon 4 and flows along the direction of the arrows. By liquefying the lining gel of cytoplasm it frees the "4" nuclei (unshaded). Similarly the "4" cytoplasm frees the "10" nuclei (shaded). The plus and minus nuclei then stream into the archicarp. The archicarp, outlined with a solid line, is an actual drawing from a 4-day-old fixed and haematoxylin-stained heterokaryotic culture. The rest of the hyphae (outlined with broken lines) are hypothetical.

a, coverslip; *b*, glass slide; *c*, Petri dish; *d*, aerial mycelium; *e*, stationary peripheral nucleus; *f*, hyphal fusion; *g*, migrating nucleus; *h*, archicarp; *i*, lining cytoplasmic layer. Magnification of Figs. 2–5, 1200.


1
A
a
B
b
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d
C
a
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g
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Mutant 4
f
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b
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f
g
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i
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4
e
Wild 10
5
h

for about an hour after it has been taken from the damp chamber. Wide trunk hyphae that are immersed in water, and that possess few if any vacuoles, are selected for examination.

Recognition of Nuclei and Nuclear Movements

In vacuolated cells, experience is required to distinguish with certainty a living nucleus from one of the smaller cytoplasmic vacuoles. Another difficulty is that vacuoles which appear to contain conspicuous spherical or elongate bodies may be acting as lenses to magnify structures which are really in the surrounding cytoplasm. For these reasons cells which have few if any vacuoles, have been selected for study.

At a magnification of about 1000 with contrast-phase illumination, the nucleus of *Gelasinospora* appears as a sphere surrounded by an amoeboid halo. The central sphere (nucleolus) is homogeneously refractile: the halo (nuclear vacuole) may contain shifting radial filaments, may have a refractile fringe at its outer margin, and sometimes has a small satellite attached to it (Fig. 6). The diameter of the nucleus is about 3 μ, that of the central body, about 1.5 μ.

To study movements of cytoplasm and nuclei, it is usually desirable to survey the field under lower magnifications (300–600), and then one can distinguish only the central bodies of the nuclei (Fig. 7). Since they show the position of nuclei, they are here referred to as nuclei, but it is understood that the chromatin resides outside them (1, 9, 10). Figs. 2–5 show the nuclei as spheres of equal diameter because the variably shaped nuclear vacuoles, which are difficult to see at low magnifications, are omitted.

Observations

(1) Control

In a 20-hour-old microculture of strain mutant 4, nuclei were distinctly visible. They were distributed against the cell walls. Lateral branches of two parallel trunk hyphae had fused at their tips and cytoplasm was flowing from one hypha, via the fused branches, into the other. The stream moved rapidly in a series of jerks carrying vacuoles with it through the septal perforations of 35 consecutive cells, a distance of about 2.5 mm. During the time it was watched (25 minutes) no nuclei appeared in the moving cytoplasmic stream and the peripheral nuclei remained in the same position.

Many similar observations were made with the same result. The streaming cytoplasm sometimes carried small spherical or elongate brightly shining mitochondria or large dark vacuoles, but no nuclei.

These negative observations support the earlier experiment work (6) suggesting that nuclei do not migrate through a homokaryon.

(2) Observation on Mated Homokaryons

In a 24-hour-old microculture that had been inoculated with strain 4 (mutant) and strain 10 (wild), which were of opposite mating type, a length

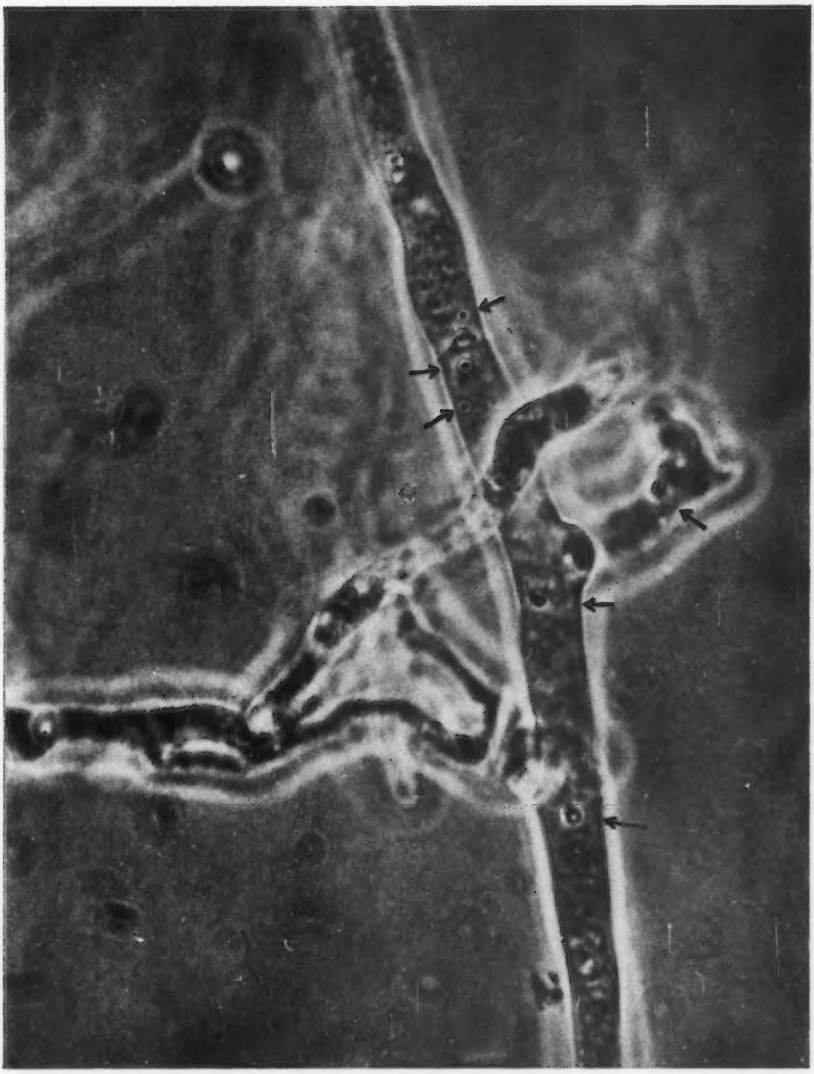

FIG. 6. Living hypha of *G. tetrasperma* with nuclei (arrows). The nuclei possess a central body and a surrounding clear area. The nuclei in which the central bodies are dark are contracted, and appear to possess a nuclear membrane. Three, in which the central body is light, are expanded and irregular in outline. Two of these three possess a small excentric sphere. Phase contrast. Magnification, 1200.

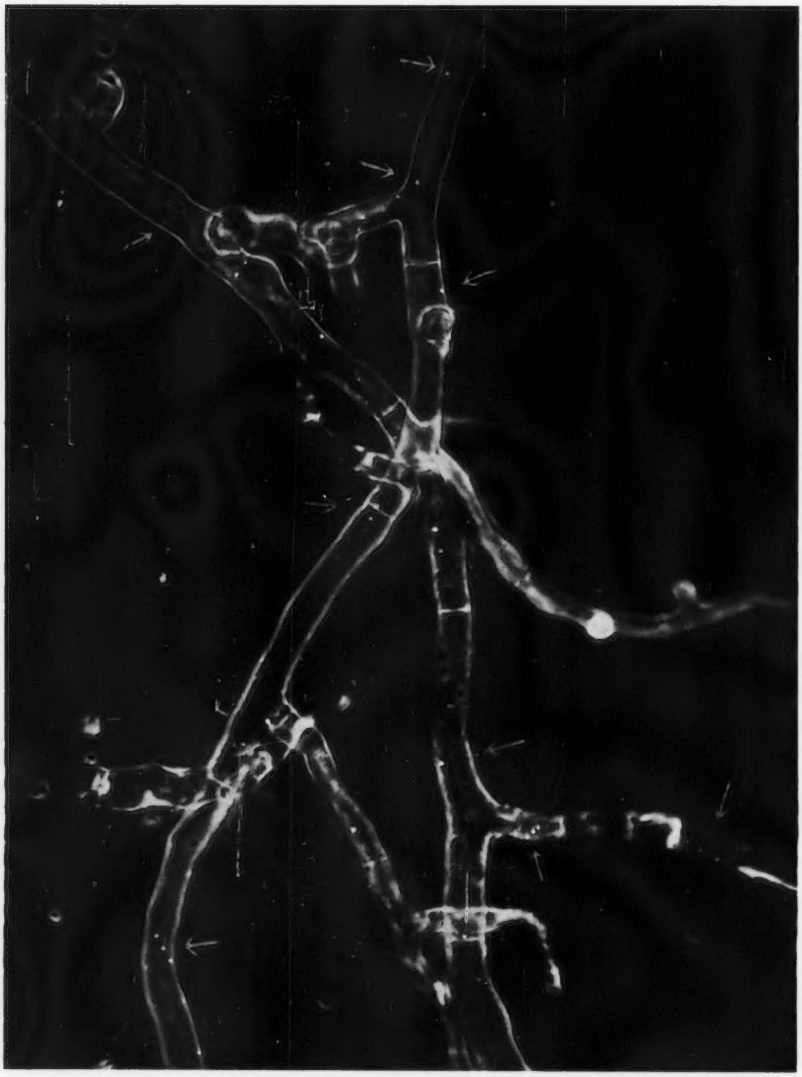

FIG. 7. Living hyphae of *G. tetrasperma* with nuclei (arrows). Only the highly refractile central bodies (nucleoli) are visible. The distribution of the nuclei in groups of three is coincidental. The hyphae appear to be almost empty because the cytoplasm lacks visible granules. The background is darker than that in Fig. 6 because the microculture is thinner. Phase contrast. Magnification, 600.

of a main hypha three cells long with four nuclei distinctly visible was chosen for observation (Fig. 2). Cytoplasm was streaming rapidly backwards from the apex, carrying vacuoles along with it. The stream crossed the field of view, a distance of 200 μ in 18 seconds. It must have been travelling about 40 mm. per hour. After a minute or two, three nuclei came into view and travelled in the center of the hypha with the cytoplasm through the three cells. Then after a time, six more close together, passed along in the same direction. Later another three nuclei were carried past. The nuclei continued to stream during the hour of watching, when the observation was discontinued. The speed of the nuclei was equal to that of the cytoplasm, about 40 mm. per hour. The four peripheral nuclei (Fig. 2) remained unmoved throughout the observation.

Similar hyphae were observed in other microcultures. In some hyphae through which nuclei migrated, there were also stationary peripheral nuclei; in others no nuclei were visible except the moving ones.

In one hypha in which the cytoplasm had been streaming slowly for about 30 minutes, a nucleus appeared and slowly passed through eight septal pores and came to rest at the ninth.

In another culture, one hypha was observed with very rapid streaming. A nucleus quickly came into view. The mechanical stage was moved rapidly to follow the nucleus as it passed through a hyphal fusion into another hypha (Fig. 3). Altogether it travelled a distance of about 19 cells. The protoplasmic streaming was filmed for about one hour, when the stream slowed down and reversed itself. A nucleus then travelled into the field of view in jerky movements. It passed through several cells, first in one direction and then in the other, until it was finally carried away by the resumption of a steady flow of cytoplasm in one direction.

In the cinephotographs of nuclear streaming, 16 nuclei in one hypha were counted during a period of 18 seconds as they travelled across the screen.

(3) The Speed of Nuclei

Nuclei have been watched as they passed along the mycelium at a speed equal to that of the cytoplasm which carried them. This speed is nearly 10 times greater than that previously estimated from indirect experimental evidence (4, 6).

The speed of cytoplasmic streaming is known to be high. Bakerspigel, working in this laboratory, found that the cytoplasm of *G. tetrasperma* streamed too rapidly to make an accurate estimate of the speed. He watched the vacuoles going through the mycelium for 23 minutes, a rate of 25 cm. per hour. Similarly the streaming of cytoplasm in Phycomycetes has been estimated as 20 cm. per hour (3).

Now nuclei have been actually timed to travel through hyphae at a rate of 40 mm. per hour. They may travel much faster.

The Mechanism of Nuclear Migration

In earlier studies of *Gelasinospora*, streaming cytoplasm was always seen to leave the nuclei behind (6). Therefore it was postulated that nuclei were carried from one mycelium into another by some unknown mechanism (4). The fact that cytoplasm carries nuclei, not only makes this postulate unnecessary, but it also explains earlier observations on the effect of light on the distribution of perithecia.

In darkened cultures of mated *Gelasinospora* strains, perithecia developed only upon the illuminated parts of the agar. The nuclei seemed to travel towards the light (6). Since, in certain Phycomycetes, light influences the direction of cytoplasmic streaming (3), we can assume that light has a similar effect on the cytoplasm, and therefore on the nuclei, of *Gelasinospora*. This is a valuable provision, because in nature, after several mycelia of *Gelasinospora* in the soil have grown out, met, and fused with one another, it is an advantage to the fungus that nuclei tend to be carried towards the light, that is, to that part of the mycelium that happens to be above ground. Thus, after plus and minus nuclei have collected and fused in pairs and the perithecia have developed, the ascospores can be discharged through the positively heliotropic perithecial necks, into the sunlight.

Although the carriage of nuclei in streaming cytoplasm has been observed, the method of their entry into this stream can only be postulated.

Marine animal eggs, muscle fibers, and cells in general, have been shown to be made up of two kinds of protoplasm, a fluid central part, and a thin rigid gel-like peripheral layer (8). In the Phycomycetes the peripheral layer is known to be distinct from the main central cytoplasmic stream. In Phycomycetes and also in *Gelasinospora* hyphae, the central part of the cytoplasm may move in one direction while the cortical part moves in the opposite direction.

In *Gelasinospora* and *Neurospora* careful microscopical examination of a hypha shows that the nuclei are usually to be found in the peripheral layer and appear to be detached from it with difficulty. The central cytoplasm, when it is in motion, jostles the peripheral nuclei about, even carrying them away for several microns. Yet they return to their original positions as though they were attached there by some elastic material. When a cell is injured, most of its contents are withdrawn but a thin lining layer of cytoplasm and nuclei remains behind (Fig. 4, *e*). Furthermore, when a hypha is increasing in length and the central cytoplasm flows slowly to the hyphal tips, the nuclei do not collect into the apical cell (6). In short, the nuclei are usually held firm in the gel-like peripheral cytoplasm even when the fluid central cytoplasm is in motion.

However, when appropriate strains are mated, the nuclei must move away from the lining gel to enter the fluid cytoplasmic stream and travel to the archicarps as shown in Fig. 5. At *h*, pairs of nuclei may be seen within the archicarp.* In the same drawing it is assumed that when "10" cytoplasm

*In this respect heterokaryotic perithecial rudiments differ from homokaryotic ones (unpublished information).

enters "4" hyphae, it acts in some way upon the lining cytoplasmic layer, changing it from a gel to a sol and freeing the "4" nuclei. The nuclei then stream in the direction of the arrows, through a second hyphal fusion and back into the "10" mycelium. Here the action of the "4" cytoplasm frees the "10" nuclei from the cell walls. By this mechanism the nuclei could be mobilized so as to collect in a mass at the base of the archicarp.

The vigorous fruiting or heterokaryotic vigor following matings between strains "10" and "4" may be due to the strong physical reaction between the cytoplasm of the two strains as compared to the weaker reaction between those strains which fruit poorly (5).

Acknowledgments

The author wishes to thank the following persons for assistance: Mr. Tatsuso Yamamoto of the University of Alberta with the photomicroscopy and Mrs. A. H. H. Grinchenko of the Canadian Department of Agriculture with the translation of scientific papers.

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NOTE ADDED IN PROOF: As this paper goes to press, Snider and Roper observed in *Schizophyllum commune* that the speed of nuclear migration in mated biochemical mutants was as much as 10 times greater than the growth of hyphal tips (Am. J. Botany. In press).

USE OF AN ELECTROLYTIC RESPIROMETER TO STUDY DENITRIFICATION IN SOIL[1]

J. W. McGarity,[2] C. M. Gilmour, and W. B. Bollen

Abstract

An electrolytic respirometer and gas-sampling attachment suitable for soil denitrification studies is described. With this apparatus, control of moisture and oxygen content in incubating soils is obtained over long periods while at the same time small quantities of gases released by denitrification are retained for estimation. Experiments using N^{15} confirmed the value of this apparatus. Only slight denitrification was found under aerobic conditions in a Walla Walla silt loam soil, and the conditions under which denitrification took place indicated the specific role of substrate level and the related gas-exchange characteristics of the system.

Introduction

Within recent years the denitrification process or nitrogen-loss problem in soil and in liquid culture media has attracted considerable attention (2, 4, 9, 16, 18). There is little doubt that nitrogen, or the oxides of nitrogen, may be evolved under anaerobic conditions. Thus in water-saturated soils containing free nitrate ions and a readily oxidizable carbon source, nitrogen losses are to be expected. However, under aerobic conditions the evidence for active denitrification either in soils or liquid media is much less conclusive and is therefore still open to conjecture. Meiklejohn (12) and Marshall *et al.* (11) have demonstrated nitrogen loss from nitrate in aerobic liquid culture media. Other investigators (9, 18) in experiments made with soil as the culture medium have shown that nitrogenous gases are evolved under aeration conditions similar to those encountered in well-drained field soils. In view of the recent study of Skerman and MacRae (14), which presents strong evidence against aerobic denitrification as such, serious doubt must arise as to the importance of denitrification in well-aerated soil systems.

The major problem in these studies appears to lie in the experimental procedures used. It is considered essential in aerobic denitrification studies that the partial pressure of oxygen above and in the test soils be maintained at a nearly constant level approaching that of the normal atmosphere; that the moisture content of the soils be maintained well below saturation values (50–60% of saturation); and that a readily oxidizable source of carbon and free nitrate ions be present. Serious experimental difficulties are involved in the exact control of these factors, particularly over long experimental periods; if control is lacking, however, it is questionable whether aerobic denitrification is under study at all.

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[2]Holder of a Thomas Lawrance Pawlett Scholarship of The University of Sydney, N.S.W., Australia.

As the direct determination of nitrogen gases evolved from soils offers the most conclusive evidence of denitrification, an attempt has been made to develop an apparatus and procedure which would allow control of partial oxygen pressure, control of moisture level, supply soil respiration data, and enable sampling of any evolved gases. Thus equal space has been devoted to a description of the method employed and of the results pertaining to denitrification.

Experimental Methods

Apparatus

An electrolytic respirometer, originally described by Swaby and Passey (15) and subsequently modified by other workers (3, 17), was redesigned to meet the needs of the present study and to give a broader application to other soil metabolic studies. A diagram of the component parts is shown in Fig. 1 and a photograph of four complete units is shown in Fig. 2. The modified apparatus so illustrated has the advantage of compactness, increased accuracy, and greater sensitivity.

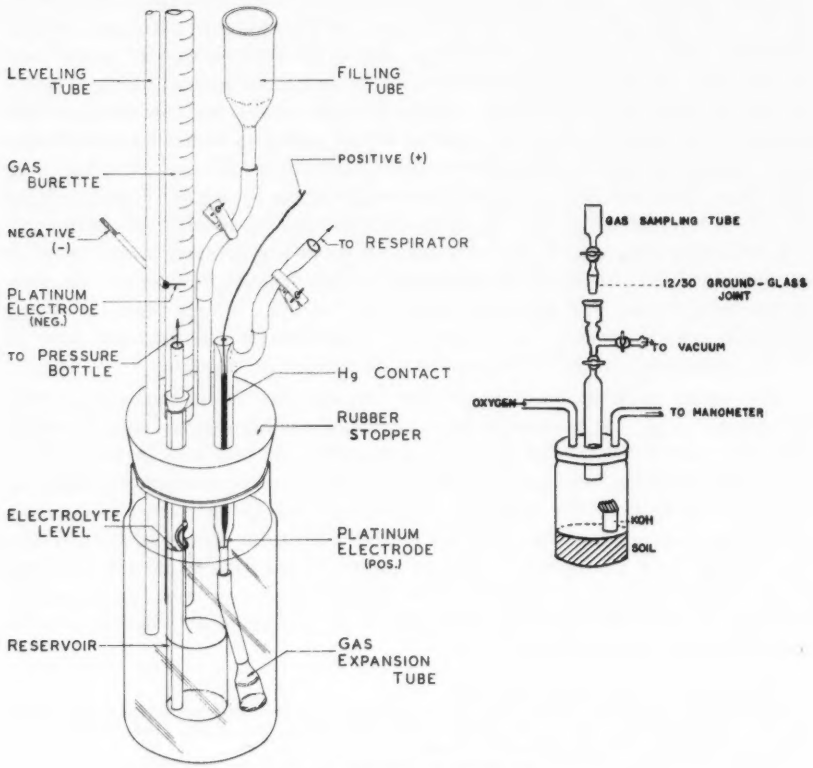

FIG. 1. Electrolytic respirometer.

The oxygen generator is composed of a 50-ml. burette which functions as the cathode, an hydrostatic leveling tube, an electrolyte filling tube, a reservoir and draining tube, and an oxygen-producing platinum anode, all of which are inserted through a five-hole rubber stopper which seals the electrolyte jar. A wide-mouthed jar of 500 ml. capacity was found ideal for compactness and stability.

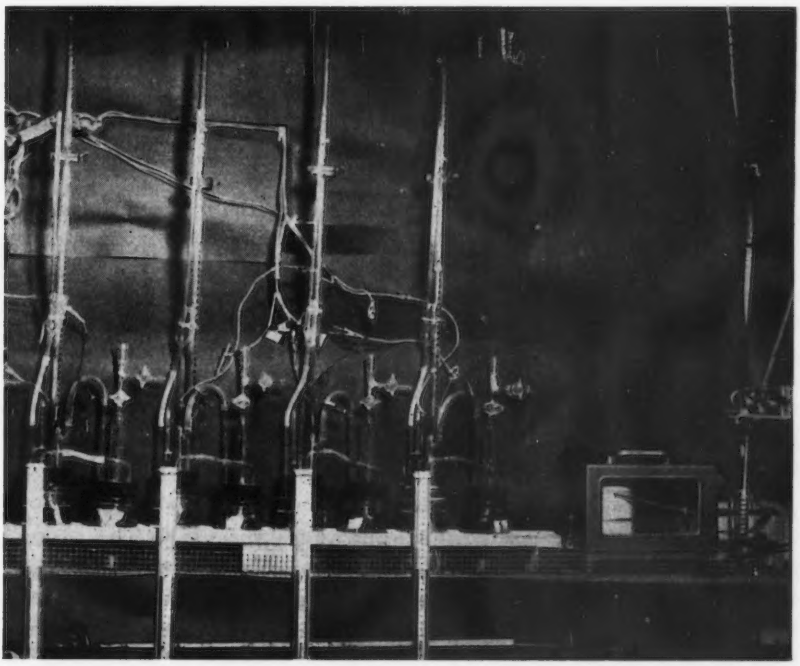

FIG. 2. Electrolytic respirometers in operation in constant-temperature cabinet.

A platinum wire sealed into the burette wall is connected to the negative terminal of a series of 12-volt wet-cell batteries. The leveling tube together with the gas burette dips into the electrolyte solution (8% Na_2SO_4) for the purpose of equilibrating the pressure within the gas burette at the time of reading.

The overflow reservoir is a glass vessel of approximately 40 ml. capacity with an off-center neck. The neck has a perforation which permits electrolyte displaced from the gas burette to spill into this vessel, thus maintaining a constant level in the generator. A thin glass tube reaching to the bottom of the reservoir permits it to be emptied as the need arises. The filling tube enables addition of electrolyte and can be sealed with a clamp.

The oxygen electrode contains an inner glass sleeve through the wall of which is inserted a platinum wire. The electrode is so constructed that small fluctuations in pressure in the respiration vessel cause immediate movement of

the contacting electrolyte, but any carry-over of electrolyte through foaming of the evolved oxygen is prevented. The battery connection to the platinum electrode is completed through mercury. A small bell-shaped glass tube connected by plastic tubing to the bottom inlet of the oxygen electrode provides a safety margin in case of gas expansion in the respiration vessel. The respiration vessel is a common fruit-preserving jar of either $\frac{1}{2}$, 1, or 2 pint capacity, the similar-sized mouths allowing ready interchange. The jar is sealed by a three-hole stopper through which is inserted the oxygen-supply inlet, the gas-sampling connection, and the manometer connection. The gas-sampling bulb of approximately 20 ml. volume fits into a 12/30 ground-glass joint. The arrangement of three stopcocks allows evacuation of the tube and sampling of the respiration-vessel gases. The apparatus in this form is set up in a constant-temperature cabinet.

The uptake of oxygen in the respiration vessel and the absorption of released carbon dioxide in 30% KOH produce negative pressure which induces upward movement of the electrolyte thus closing electrical contact at the anode. The evolution of oxygen continues until normal pressure is attained and the contact broken. The volume of oxygen released is equal to half the volume of hydrogen collected in the gas burette and can be recorded at regular intervals. The nearly continuous flow of oxygen prevents escape of jar atmosphere gases into the electrolyte. Small quantities of gases released from the soil during the incubation period are trapped in the respiration vessel; herein lies the particular value of this apparatus in denitrification studies.

Procedure

The oxygen generator is partially filled with solution of electrolyte. With both the oxygen outlet tube and filling tube clamped, the gas burette is opened and the burette is filled with electrolyte by pressure applied through the reservoir drain tube. The filling tube is next opened and electrolyte added until the level reaches the opening in the neck of the overflow reservoir. Any electrolyte spilled into the reservoir can be removed by suction.

The respiration vessel can be filled with varying quantities of soil and different substrates, but an oxygen consumption of approximately 1–2 ml. per hour is convenient for long-term experiments. The soil is brought to the conditions of moisture and substrate content required by mixing in the vessel. The temperature of all these constituents should be close to that of the experimental conditions in order to shorten the time for equilibration. Finally, a small vessel containing 10 ml. of 30% KOH and a pleated filter paper is placed in the respiration vessel. The rim of the KOH vessel is covered with a film of alkali-resistant grease to prevent creep of the solution into the soil.

The stopper is placed on the respiration vessel and sealed with wax with all connections open. The respiration vessel is attached to the manometer and to the connecting tubing from the oxygen electrode; the clamp on the latter connection can now be opened. After equilibrating for 1 hour, the stopcock on the gas-sampling tube is closed. The procedure for taking readings and

refilling gas burettes is similar to that described above. The tube connecting the oxygen electrode to the respiration vessel should first be closed, the filling tube clamped, and the electrolyte raised in the leveling tube to the same level as that in the gas burette. This is done by forcing air into the unit through the reservoir drain tube. The reading can then be made and the burette refilled. The amperage and voltage of the direct current should be such that the rate of oxygen release is not limited.

Gas samples can be taken at intervals during the experiment. The gas-sampling bulb is evacuated and with the oxygen inlet closed the stopcock leading to the respiration vessel may be opened. The stopcocks are then closed and the gas bulb removed. The pressure difference in the vessel is read from the manometer and then adjusted to the initial pressure with gas of known composition, usually that originally used. As both the volume of the respiration vessel, gas bulb, and joint are known, a recalculation of the gas composition of the respiration vessel can be made. When final readings and gas samples have been taken, the vessel is opened, and KOH is removed and washed into a stoppered bottle with CO_2-free water. The incubated soil can then be removed for determination of moisture; pH; nitrate, nitrite, and ammonia nitrogen; total nitrogen; and soluble carbon.

Soil Treatment

The surface 6 inches of Walla Walla silt loam, a chestnut soil from eastern Oregon, with pH 6.2, was used throughout the study. The soil was collected from a fallow wheat field, air-dried, and stored in bins.

In the first experiment, wheat straw (0.35% N) ground to 80 mesh, and potassium nitrate enriched with 29.845 atom % N^{15} were added to 100 g. of air-dried soil and incubated at 30° C. and 31.8% moisture (60% saturation) for 7 days in the respirometer. Optimum crumb structure was established during addition of water. The treatments are listed in Table I. Oxygen consumption was followed over this period for all treatments and CO_2 evolved and absorbed by the KOH was determined by differential titration at the end of 7 days. A nitrogen balance was made at the beginning and at the end of the experimental period. Denitrification of added N^{15} was determined by mass spectrometer analysis of the gas atmosphere, and as a further check an analysis was also made of N^{15} remaining in the soil sample.

The second experiment was divided into aerobic and anaerobic sets. One set was incubated in the respirometer under aerobic conditions and the other in sealed respiration vessels with the total oxygen supply limited to 20% of the vessel volume. As this oxygen was rapidly depleted, the treatment has been termed anaerobic. Wheat straw and enriched potassium nitrate additions were made to 100 g. soil, dry basis, in each set and the incubation continued for 14 days at 30° C. and 60% saturation. The treatments are shown in Table II. The initial and final analyses were made as above but no attempt was made to recover N^{15} from the soil.

Nitrate and nitrite nitrogen were determined on a 4:1 aqueous extract of soil by a modified phenoldisulphonic acid method (6) and the Griess reaction (7) respectively. Ammonia nitrogen was determined by nesslerization of a distillate of a 4:1 extract of soil made with N sodium chloride.

The salicylic acid modification of the micro-Kjeldahl method (13) was used for total nitrogen. The macro method was used to obtain 5–10 mg. samples of ammonium sulphate for determination of the isotope enrichment. Carbon dioxide determinations were made by the double titration method (5) using the Beckmann automatic titrator; soluble carbon was determined by a wet-persulphate oxidation.

Analyses were made in duplicate except for the single N^{15} determinations of nitrogen recovered from the soil. The estimated error of the gas analyses values was ± 0.5 μg. NO_3-N per g. of soil. These determinations were carried out using a Consolidated Nier-type mass spectrometer. The gas analyses have been expressed on the basis of μg./g. oven-dried soil (105° C.) to allow for better comparison with the other soil nitrogen data. The micro-Kjeldahl nitrogen determinations were carried out in triplicate.

Results

The type of atmosphere-control data which may be obtained with the electrolytic respirometer is shown in Table I. The experiments termed "aerobic" had the electrolysis unit attached to the respirometer containing incubated soil whereas the "anaerobic" experiments did not. The final moisture and oxygen content of the soil samples and vessel atmosphere respectively indicate clearly the value of the apparatus in maintaining control of these factors. The slight disparity between initial and final oxygen levels appears to be due to an increase in partial pressure of water vapor and perhaps to an initial lag in temperature equilibration.

The aerobic Q_{O_2} values, ranging from 1.35 with the non-treated soil to 12.42 with the wheat straw and nitrate-treated soil, compare favorably with those obtained by means of the Warburg respirometer and reported in an earlier paper (8). It is felt, therefore, that the apparatus supplies soil-respiration data essentially similar to that obtained by manometric methods.

As might be expected, wheat straw and nitrate additions produced an overall rise in oxygen consumption and carbon dioxide evolution under aerobic conditions (Figs. 3 and 4). In addition, the inclusion of nitrate nitrogen brought about an increase in rate of oxidation of wheat straw up to 121 μg./g. nitrate nitrogen level. At the 221 μg./g. level of nitrate a slight decrease in rate of oxygen consumption was observed. It is noteworthy also that under aerobic conditions the R.Q. values consistently remained close to 1.0 with both the treated and non-treated soil. By comparison, low rates of oxygen consumption and carbon dioxide evolution were recorded in all soil samples with substrate additions when incubated under anaerobic conditions.

When added to fallow soil, nitrate nitrogen may temporarily be immobilized in the form of bacterial protein, undergo leaching, or be reduced to a gaseous

TABLE I

TYPICAL SOIL RESPIRATION DATA OBTAINED BY MEANS OF THE ELECTROLYTIC RESPIROMETER

Soil treatment*	Time, hours	μl./g./hour CO_2 evolved	μl./g./hour O_2 consumed	R.Q.	Final† O_2, %	Final† H_2O, %
Aerobic (1)						
None	164	1.09	1.35	0.81	20.1	25.8
1% W.S.	164	7.44	8.24	0.90	19.2	27.8
1% W.S.+ 27.5 μg. NO_3-N	164	9.62	9.38	1.03	17.3	27.3
1% W.S.+ 52.5 μg. NO_3-N	164	10.83	10.10	1.07	17.7	26.8
1% W.S.+102.5 μg. NO_3-N	164	12.65	11.65	1.09	18.1	27.0
1% W.S.+202.5 μg. NO_3-N	164	9.60	9.01	1.07	18.7	27.5
Aerobic (2)						
1% W.S.+200 μg. NO_3-N	324	8.01	7.35	1.09	17.8	28.3
1% W.S.+200 μg. NO_3-N	324	7.66	7.38	1.04	18.4	28.0
2% W.S.+400 μg. NO_3-N	324	12.55	11.92	1.05	n.d.	28.2
2% W.S.+400 μg. NO_3-N	324	12.68	12.42	1.02	17.6	28.8
Anaerobic‡						
1% W.S.+200 μg. NO_3-N	324	3.20	1.40§	2.30	0.3	27.6
1% W.S.+200 μg. NO_3-N	324	3.13	1.40	2.25	0.3	28.4
2% W.S.+400 μg. NO_3-N	324	4.38	1.40	3.14	0.2	28.9
2% W.S.+400 μg. NO_3-N	324	4.52	1.40	3.24	0.2	26.8

*KNO_3 enriched with 29.845 atom % N^{15}; added on basis of μg./g. oven-dried soil. Wheat straw abbreviated to W.S.
†Initial per cent O_2 = 20.1%; initial per cent H_2O in soil = 31.8% (60% of saturation).
‡Oxygen supply limited.
§Calculated from vessel volume.

state via the denitrification process. Leaching may be ignored in the present study. The data shown in Table II provide needed information relative to the amount of mineral nitrogen which is quickly assimilated by the soil flora and thereby is not subject to rapid loss via denitrification. After 164 hours of aerobic incubation the lower levels of added nitrate nitrogen disappeared, but on the basis of total nitrogen and isotope nitrogen recoveries, remained in the soil in some other form. When 221 μg. of nitrate nitrogen was present at the start of the experiment, however, a considerable part (153 μg.) remained after 7 days' incubation. In the latter instance, it may be concluded that for the time period indicated, microbial assimilation of added nitrate for protein synthesis has been partially satisfied at least, and that the remaining free nitrate could be subject to denitrification. In addition, soluble carbon as well as oxygen consumption values confirmed the presence of remaining carbohydrate substrate. Under the aerobic conditions employed, no nitrogen losses were observed. Recoveries calculated on the basis of total nitrogen and isotope beam ratios accounted for all of the added nitrate. Then of equal significance, no N^{15} enrichment of the vessel atmosphere could be detected.

These results determined the planning of subsequent experiments. On the basis of the previously observed microbial assimilation and immobilization of added nitrate nitrogen, 200 and 400 μg. levels of added nitrate were considered appropriate for denitrification studies. For purposes of comparison, anaerobic

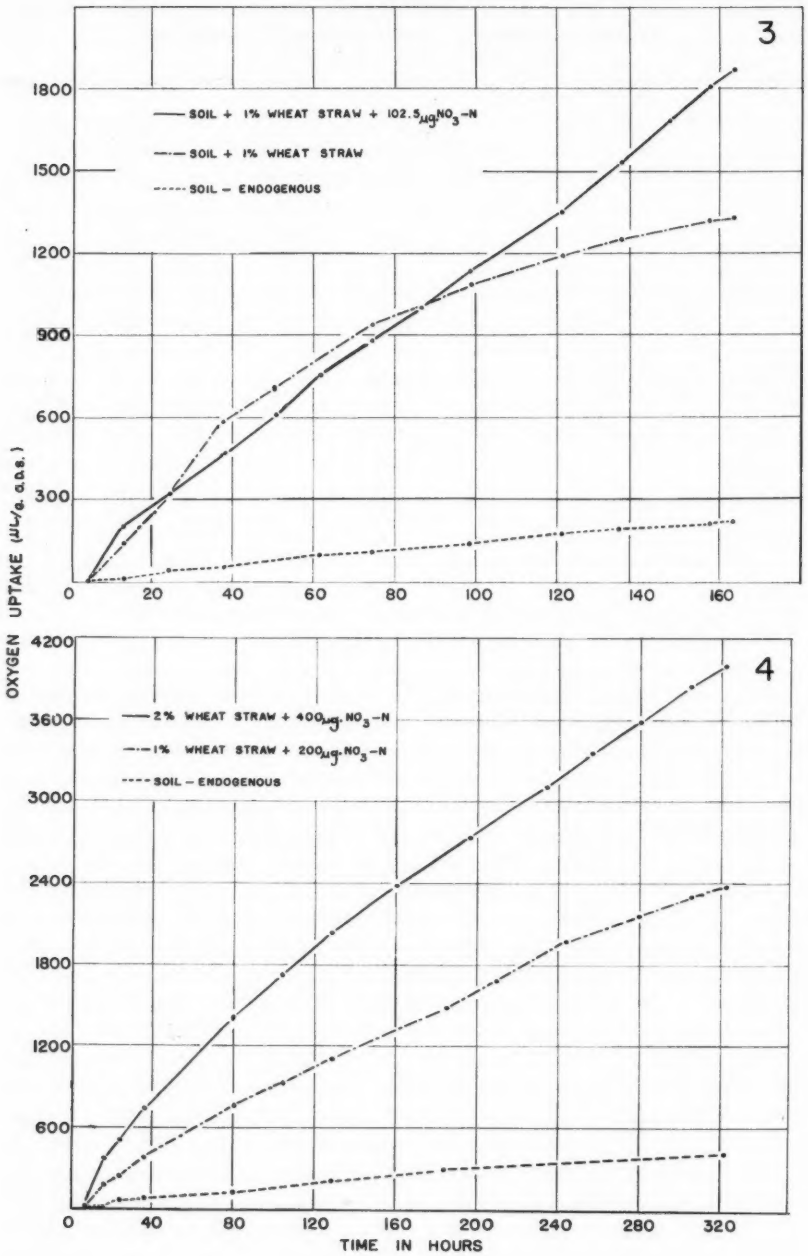
3
SOIL + 1% WHEAT STRAW + 102.5 µg NO3-N
SOIL + 1% WHEAT STRAW
SOIL – ENDOGENOUS
OXYGEN UPTAKE (µl/e. a.a.s.)
1800
1500
1200
900
600
300
0
20
40
60
80
100
120
140
160
4
2% WHEAT STRAW + 400 µg. NO3-N
1% WHEAT STRAW + 200 µg. NO3-N
SOIL – ENDOGENOUS
4200
3600
3000
2400
1800
1200
600
0
40
80
120
160
200
240
280
320
TIME IN HOURS

TABLE II

NITROGEN TRANSFORMATIONS AND BALANCES IN WALLA WALLA SILT LOAM INCUBATED UNDER AEROBIC CONDITIONS

Soil treatment	Time, hours	Mineral N, μg./g.			Total N, μg./g.*	Recovery (N^{15}), μg./g.	
		NH_4^+	NO_2^-	NO_3^-		Gas	Soil
None	0	10	0	18	1022	–	–
	164	15	0	18	1026	–	–
1% wheat straw	0	10	0	18	1061	–	–
	164	2	0	8	1050	–	–
1% wheat straw + 27.5 μg. NO_3-N	0	10	0	46	1082	–	–
	164	1	0	5	1071	0	30.7
1% wheat straw + 52.5 μg. NO_3-N	0	10	0	71	1108	–	–
	164	4	0	2	1104	0	52.7
1% wheat straw + 102.5 μg. NO_3-N	0	10	0	121	1156	–	–
	164	6	0	8	1169	0	103.1
1% wheat straw + 202.5 μg. NO_3-N	0	10	0	221	1251	–	–
	164	9	0	153	1241	0	202.4

*μg./g. refers to μg./g. oven-dried soil.

experiments were included alongside those termed aerobic and the incubation time was extended to 14 days. Typical data from these studies are given in Tables III and IV. Duplicate runs are shown at the 324-hour time interval. It is quite evident that very little gaseous loss took place in the soils incubated under aerobic conditions. These denitrification losses ranged between 0.1 and 4.2% of the initial nitrate present in the soil. Inasmuch as these denitrification values are based on the N^{15} isotope content of the final vessel atmosphere, they are far less subject to experimental error than the denitrification losses calculated from the differences in Kjeldahl total nitrogen levels at the beginning and end of the experiments.

A further study of the data in Table III discloses that under anaerobic conditions a considerable amount of the added nitrate nitrogen escaped via the gaseous route. These losses ranged from 44.9% of the initial nitrate nitrogen in the lower nitrate and wheat straw addition, to 66.7 % at the higher treatment level. In contrast to the aerobic set, all free nitrate disappeared from the soil and a marked rise in ammonia nitrogen was observed. The increase in ammonia nitrogen of the soil may have resulted from either a direct reduction of nitrate to ammonia or continued ammonification of organic nitrogen in the absence of nitrification.

FIG. 3. Oxygen consumption of soil measured by the electrolytic respirometer over a period of 7 days.

FIG. 4. Oxygen consumption of soil measured by the electrolytic respirometer over a period of 14 days.

TABLE III

INFLUENCE OF OXYGEN, WHEAT STRAW, AND NITRATE ON DENITRIFICATION IN WALLA WALLA SILT LOAM

Soil treatments	Time, hours	Mineral N, μg./g. NH_4^+	NO_2^-	NO_3^-	Rec.* gas (N^{15}), μg./g.	Total N, μg./g.	Total N rec., %	Denit.,† %
Aerobic								
1% wheat straw	0	10	0	218	–	1262	–	–
+	324 (1)	15	8	123	8.9	1233	98.4	4.1
200 μg. NO_3-N	324 (2)	14	7	114	9.2	1237	98.8	4.2
2% wheat straw	0	10	0	418	–	1500	–	–
+	324 (1)	9	0	230	0.3	1470	98.0	0.1
400 μg. NO_3-N	324 (2)	9	1	230	5.4	1460	97.7	1.3
Anaerobic‡								
1% wheat straw	0	10	0	218	–	1262	–	–
+	324 (1)	32	0	0	101.0	1099	95.1	46.4
200 μg. NO_3-N	324 (2)	31	0	0	98.0	1097	94.7	44.9
2% wheat straw	0	10	0	418	–	1500	–	–
+	324 (1)	31	1	5	264.0	1166	95.3	63.2
400 μg. NO_3-N	324 (2)	30	1	0	279.0	1175	96.9	66.7

*Recoveries calculated from N^{15} isotope data obtained on respective gas samples of atmosphere above the test soil.
†Denitrification losses calculated from N^{15} gas recoveries as a percentage of the initial nitrate level.
‡Limited oxygen supply.

TABLE IV

NATURE OF GASES FORMED FROM SOIL NITRATE

Soil treatment	Gas as μg. N/g. soil* N_2O	N_2
Aerobic†		
1% W.S.+200 μg. NO_3-N	8.5	0.6
2% W.S.+400 μg. NO_3-N	1.8	1.1
Anaerobic‡		
1% W.S.+200 μg. NO_3-N	0.0	99.8
2% W.S.+400 μg. NO_3-N	0.0	271.7

*Gas recoveries calculated from N^{15}, scan and isotope ratio.
†Wheat straw.
‡Limited oxygen supply.

The nature of the gases evolved during denitrification contributes to our knowledge of the chemical process itself. In the present investigation, mass spectrometer scan analysis of the final gas mixture in each vessel was obtained. Two such sets of scan data are shown in Fig. 5. The aerobic and anaerobic spectrum runs are shown on a single chart to enable better comparison of the results. Mass peaks below 28 were omitted since soil gases such as hydrogen, ammonia, or methane were not observed in these scan areas. It is immediately apparent that with an adequate concentration of free oxygen, very little mass 29 or 30 in excess of natural abundance was detected in the jar atmosphere; whereas at an approximately zero oxygen

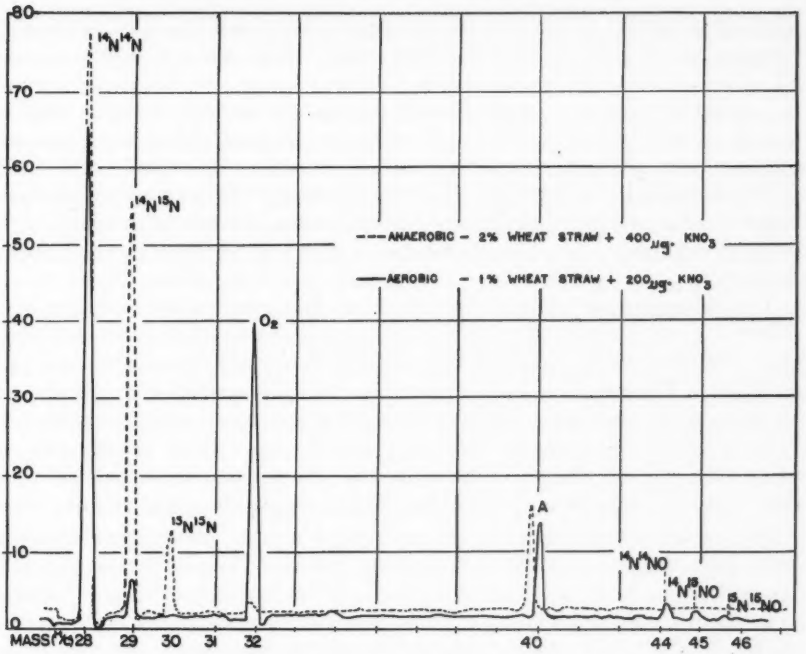

FIG. 5. Mass spectrometer scan analysis of gas atmosphere from respiration vessel after 14 days.

pressure, relatively high levels of mass 29 and 30 were observed. Of additional interest are the small peaks observed at mass 44 and 45 with the aerobic set. Since carbon dioxide was removed from the vessel atmosphere, and since the heavy nitrogen isotope appeared at mass 45, it was concluded that nitrous oxide had been evolved. No such peaks were observed in the absence of molecular oxygen where the only evolved gas detected was nitrogen. Thus, as depicted in Table IV, small quantities of nitrogen and nitrous oxide were evolved in the presence of free oxygen, whereas a comparatively large quantity of nitrogen only was found under anaerobic conditions. The pH of the soils under anaerobic conditions rose to 7.2–7.6 due to the released K^+ ions from the added KNO_3. These results are in general agreement with other workers (9, 18) who have shown molecular nitrogen to be the predominant gas evolved under anaerobic conditions.

Discussion

Over the years a variety of experimental techniques has been used to study the denitrification process in soil. The conclusions which have been drawn from the experimental data so obtained have been based on either indirect measurement of loss by difference of total nitrogen levels at the beginning and end of experiments, or the direct determination of gases evolved. Indirect

methods generally utilize the Kjeldahl procedure modified for inclusion of nitrates, but it is recognized that this method does not completely recover all mineral forms of nitrogen which may be present. In Kjeldahl analyses performed prior to the present study, an average recovery of only 93% of nitrate added to the Walla Walla silt loam was obtained, although the amount of this nitrate nitrogen plus the original soil nitrogen represented nearly 99% of the calculated amount of total nitrogen present. Thus the small nitrogen losses which occur under aerobic conditions in soils, and which may have agricultural significance, could not be measured with any certainty by the indirect method.

The determination of losses by the direct method presents problems of a different kind; these problems arise from the need to enclose the soil so that the gases liberated by denitrification do not escape but can be collected and analyzed. The usual enclosed respiration vessels described in the literature for these direct studies fall short of the basic requirements of constant control of such factors as oxygen or moisture content. In addition, small amounts of evolved gas are often so diluted by the atmosphere above the soil that detection and measurement would be difficult even with special techniques.

The electrolytic respirometer and gas-sampling attachment described offers many advantages in aerobic denitrification studies. These advantages include the maintenance of a nearly uniform oxygen and moisture level and a means of measuring the rate of oxygen consumption over long periods for large samples of soil. In addition, the volume of the respiration vessel is kept small in relation to the size of the soil sample for the reason that the smaller the volume of contacting atmosphere present, the smaller becomes the dilution effect on the gases released by denitrification. The subsequent analysis of the atmosphere enables a much more accurate determination of the accumulated gases.

The results presented above (Table I) indicate only small variation in oxygen and moisture contents, while the duplicate gas-exchange data show a high reproducibility. Under the experimental conditions described, the estimation of as little as 100 μg. of nitrogen evolved as gas per 100 g. of soil has been made by the use of isotopic nitrogen. It should be noted that the liberation of nitrogen gases slightly decreases the content of oxygen, but this can be controlled by the experimental design. Thus in the experiments reported here, the calculated decrease in the mole percentage oxygen content of the respiration vessel by the liberation of 100 μg. of nitrogen as gas would be approximately 0.03%.

The arrangement of stopcocks and manometer has also provided a means for easy gas evacuation and for filling with any desired gas mixture, and the use of the apparatus can therefore be expanded to include other methods of gas analysis (e.g., gas chromatography). It is felt, therefore, that the described apparatus offers distinct advantages not only in aerobic denitrification studies but in other studies involving gas exchange in soils.

Several features of the experimental results need further comment. Of note in the 7-day experiments were the observations that with low levels of nitrate

(121 μg. N/g. of soil or less) in the presence of 1% wheat straw, the nitrate was nearly completely immobilized within 7 days, and during this time no denitrification occurred, although a considerable amount of carbon underwent oxidation. At the highest level of nitrate (221 μg. N/g.), an excess of free nitrate remained over that immobilized by the wheat-straw decomposition at the end of both the 7-day and 14-day incubation period. Slight denitrification was observed only during the longer period. It is therefore evident that a 7-day denitrification lag existed under the conditions of these experiments. Under anaerobic conditions in this soil, active denitrification may occur within 1 day (8). As both nitrates and oxidizable carbon levels were adequate, the lag is apparently due to a delay in the development of some other condition favorable for the denitrification process.

Although the nitrogen losses reported here are extremely small, the results confirm the view held by other workers (4, 18) that denitrification can proceed in soils which are normally considered well aerated. It is questionable, however, whether the reported nitrogen losses can be considered as occurring under truly aerobic conditions, despite the fact that a high and constant oxygen concentration was maintained above the incubating soil in the apparatus. It is generally agreed that within the soil there exist small volumes in which the free exchange of gases by diffusion is restricted. These volumes, under conditions of rapid O_2 uptake and CO_2 evolution, would be more readily subject to anaerobiosis than the larger, more open soil pores. In this regard, Jansson and Clark (10) consider that with biologically active systems, a mixture of anaerobic and aerobic spots may develop in the presence of high oxygen concentration. Recent work (14) has demonstrated that active denitrification of nitrate occurs only when the oxygen supply is extremely depleted. These considerations suggest that both the activity of the soil microorganisms as influenced by substrate, and the air permeability, which is governed by such factors as moisture content, structure, and the depth of soil, would determine the final oxygen concentration.

The decreased nitrogen gas evolution and nitrite accumulation observed under aerobic conditions with the higher level of wheat straw (2%) and NO_3-N (400 μg.) as compared with the lower substrate level (1% wheat straw, 200 μg. NO_3-N) supports the above view (Table III). The presence of 2% wheat straw at the time of addition improved the air permeability by increasing both structural aggregation and moisture-holding capacity, so that although the O_2 uptake at the higher substrate concentration is 12 μl./g./hour as against 7 μl./g./hour for the lower substrate level (Table I), anaerobic effects become less important at the higher substrate level.

It appears, therefore, that the criterion for denitrification in well-drained field soils may not rest simply on the presence of nitrate in excess of that required for assimilation by the microflora. But rather, that in the presence of nitrate, a certain critical rate of oxygen uptake must be exceeded, this rate differing for each soil possessing different characteristics of structure and permeability. The small nitrogen losses occurring under a high partial

pressure of oxygen above the soil should therefore be regarded as losses resulting from oxygen depletion within the soil. In this sense the term aerobic denitrification can hardly be used.

The experiments with Walla Walla silt loam soil have been carried out at moisture levels slightly in excess of field capacity and approaching the maximum likely to occur under normal field conditions. In addition, good crumb structure has been attained under the described experimental conditions. Consequently, it appears that in wheat fallow soils of this type, under conditions of high field moisture content and good tilth, denitrification will take place only to a limited extent at specific levels of wheat straw and nitrate content. It is evident, however, that studies on other soil types and substrates are needed before the exact conditions for denitrification in well-drained soils can be further evaluated.

Acknowledgment

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NOTES

PRODUCTION OF EXTRACELLULAR POLYSACCHARIDES BY MUCOR RACEMOSUS[1]

S. M. MARTIN

In the report of a survey of the constituents of fungal polysaccharides (5) it was noted that changing the cultural conditions produced quantitative and perhaps qualitative changes in the polysaccharide material. No information was available, however, on the changes which occurred during the growth of a culture. Thus, the present study was undertaken to gain information on the changes in the constituents of the polysaccharide material during growth of the *Mucor racemosus*.

Eight replicate cultures (250 ml. each) of *M. racemosus*, N.R.C. M-96, were grown in a glucose medium under the conditions described by Martin and Adams (5). Duplicate cultures were harvested at 3, 5, 7, and 10 days (approximately 15, 35, 60, and 85% glucose utilization respectively). The mycelium was removed by filtration, washed, dried, and weighed. Residual sugar was estimated by the method of Nelson (3). The culture filtrates were fractionated by ethanol precipitation and the polysaccharide preparations were dissolved and reprecipitated as previously described (5). Aliquots of the eight polysaccharide preparations were hydrolyzed with 2 *N* sulphuric acid for 16 hours at 100° in sealed tubes. The hydrolyzates were neutralized with barium carbonate, centrifuged, spotted on Whatman No. 1 filter paper (10, 20, 30, and 40 μl. aliquots) and chromatographed with pyridine – ethyl acetate – water (1:2:2) as the solvent (2). The developed chromatograms were stained with silver nitrate and scanned in a recording integrating densitometer* as outlined previously (4). The densitometer readings, after conversion to glucose equivalents, were used to calculate the percentage of total sugar (glucuronic acid not included) contributed by each sugar.

The amount of residual sugar, mold mycelium, and extracellular polysaccharide material in the cultures at times of harvest is shown in Fig. 1. After 10 days' incubation approximately 85% of the sugar had been metabolized with the concomitant conversion of approximately 1.25% of the sugar to polysaccharide. Chromatography of the hydrolyzates of the polysaccharide preparations demonstrated the presence of fucose, galactose, glucose, and mannose and electrophoresis demonstrated the presence of glucuronic acid and an unidentified negatively charged component ($R_{\text{glucuronate}} = 2.3$) which reduced silver nitrate (X_1). In an attempt to identify X_1, 23 acids were tested but none were found to be identical.

[1]Issued as N.R.C. No. 4701.
*Analytrol, Spinco Division, Beckman Instruments Inc., Belmont, California.

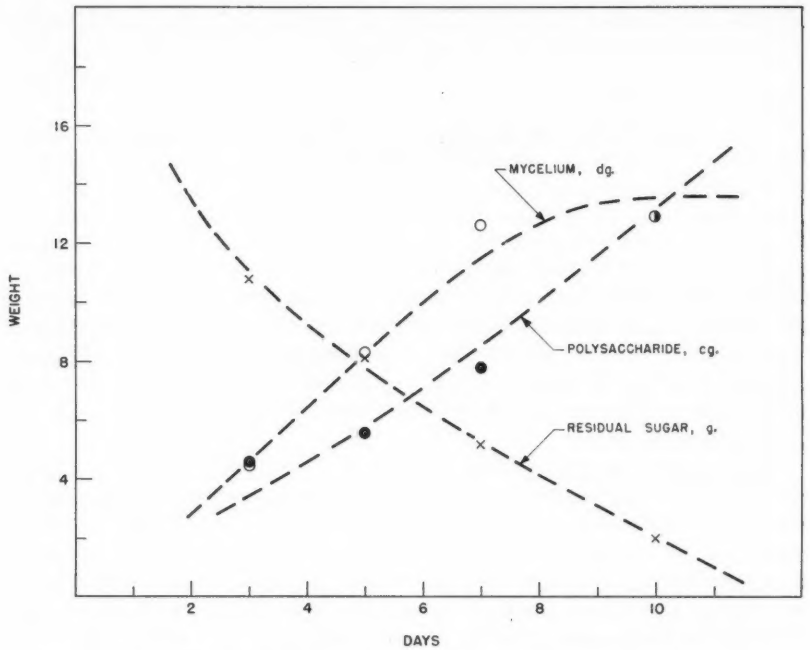

FIG. 1. Changes in amount of residual sugar, mold mycelium, and extracellular polysaccharide during incubation.

Although qualitative differences were not apparent quite pronounced changes in the relative amounts of the sugar constituents of the polysaccharide material occurred as incubation progressed. The percentage of the total sugar (glucuronic acid excluded) of the polysaccharide contributed by the individual sugars is shown in Fig. 2 as a function of incubation time. At 3 days the ratios of the sugars one to another were glucose:fucose:mannose:galactose::1.0:0.9:0.5:0.2, whereas at 10 days they were 1.0:8.3:8.0:2.6. Extrapolation of the curves in Fig. 2 would suggest that in the very early stages of growth only glucan was present whereas little or no glucose was to be found in the polysaccharide when substrate utilization was complete. This probably means that there is a gradual shift in the type of polysaccharide produced, perhaps with the concomitant utilization of some of the glucans already formed.

The extreme susceptibility of molds to slight changes in environmental conditions (1) can often account for difficulties in reproducing results from other laboratories or even between trials in a single laboratory. From the results of the present study it is seen that even in a single culture gross differences may occur in the polysaccharide material produced, depending on the age of the culture.

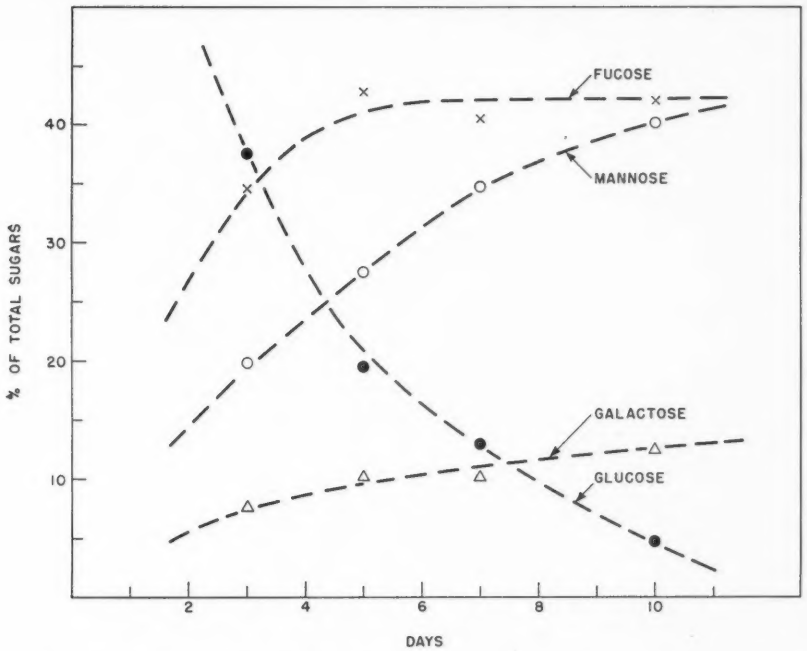

FIG. 2. Percentage of total sugar of the extracellular polysaccharide contributed by individual sugars.

Acknowledgment

The technical assistance of Mr. G. D. Powers is gratefully acknowledged.

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